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Original Article

Expression of aryl hydrocarbon receptor in relation to p53 status and clinicopathological parameters in breast cancer

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Abstract: The aryl hydrocarbon receptor (AhR) is a ligand activated transcription factor implicated in multiple cellular processes and its expression has been shown to play a critical role in tumorigenesis. However, the role of AhR in tumorigenesis of breast cancer remains unclear. In the current study, we investigated the expression levels of AhR in breast lesions and assessing the correlation between AhR expression and clinicopathological variables using breast cancer tissue microarray. Meanwhile, 10 paired of fresh breast cancer and corresponding non-cancer samples were detected for AhR and p53 expression by Western blot, respectively. Results showed that AhR expression levels in breast cancer tissues were significantly higher than that in the non-cancer tissues. AhR expression was associated with the pathological type and P53 status, but not patients age, tumor grade and TNM, as well as ER, PR, C-erbB2, Ki-67, AR, EGFR status. Moreover, Western blot data suggested a negative correlation between p53 protein and AhR protein expression levels. The results suggest that high levels of AhR were expressed in the majority of breast cancer tissues and closely associated with P53 status and histological types of breast cancer. AHR and its abnormal expression may play an important role in multiple stages of breast cancer progression.

Keywords: AhR, breast cancer, tissue microarray, p53, immunohistochemistry

Introduction

Breast cancer is the most common malignancy in women worldwide, and is the second leading cause of cancer-related mortality in women in the United States [1]. In the last two decades, the incidence and mortality of breast cancer have climbed sharply in China [2]. Historically, breast cancer emerges by a multistep process which can be broadly equated to transformation of normal cells via the steps of hyperplasia, premalignant lesions and in situ carcinoma, invasive carcinoma which supported by evidences from clinical, pathological, and genetic studies [3]. Therefore, the development of an effective target for diagnosing and treating human breast cancer is of paramount importance.

Recently, aryl hydrocarbon receptor (AhR) has been proved that multiple factors were involved in different types of cancers, such as liver cancer, lung cancer, ovarian cancer, etc [4-7]. AhR was initially identified as a ligand-activated transcription factor of the basic helix-loop-helix (bHLH) Per-Arnt-Sim (PAS) family, which could be activated by various environmental xenobiotic toxic chemicals such as benzo[a]pyrene (polycyclic aromatic hydrocarbons) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (dioxin). Recent animal and human data suggested that AhR is involved in various signaling pathways critical to cell normal homeostasis, such as cell proliferation and differentiation, gene regulation, cell motility and migration, inflammation and others [8, 9]. Dysregulation of these physiological processes is known to contribute to events such as tumor initiation, promotion, and progression [10]. Recently, AhR was observed highly expressed in the breast cancer, implied that AhR and AhR-regulated gene batteries might have potential function in human breast cancer progression [11]. The importance of some molecular markers in breast cancer has been of considerable interest during recent years,

Table 1. Procedures for evaluation of AhR expression

Percentage of positive cells	IRS (Immunoreactive Score)		Points
	Points	Intensity of reaction	
No positive cells	0	No reaction	0
<25% positive cells	1	Weak color reaction	1
25–50% positive cells	2	Moderate intensity	2
51–75% positive cells	3	Intense reaction	3
>75% positive cells	4		

including estrogen receptor (ER), progesterone receptor (PR), human epidermal receptor 2 (HER2), Ki67 and p53, etc [12, 13]. These molecular markers are not only as prognostic markers, but also as predictors of response to therapy. However, the relationship of AhR with these markers status as well as other clinicopathological variables has not been well elucidated.

In this study, the expression levels of AhR in 220 breast cancer and 8 non-cancer breast tissues were evaluated by tissue microarray and immunohistochemistry. And the association of AhR expression with clinicopathological variables was analyzed. Moreover the protein levels of AhR and p53 expression in 10 paired breast cancer and adjacent non-cancer breast tissues were evaluated by western blot, in an attempt to further investigate the functional significance of AhR in breast carcinogenesis and to explore its potential role in breast cancer treatment.

Materials and methods

Breast cancer tissue microarray

The breast cancer tissue microarray (# BRC-2281A, Pantomics, Inc. Richmond, CA) contains 8 cases of normal/benign conditions and 220 cases of breast cancers with grading, TNM staging, and IHC data of AR, ER, PR, HER2, P53, EGFR, Ki67. Each microarray tissue was organized with diameter 1 mm, thickness 5 μm. Of which 220 cases of malignant breast tumors, the major histological types were: 17 cases of ductal carcinoma in situ, 178 cases of invasive ductal carcinoma, 13 cases of invasive lobular carcinoma, 12 cases of other cancers. Clinical stage were on the basis of the sixth edition of the American Joint Committee on Cancer (AJCC) TNM criteria. Two microarrays were run in parallel: one was probed with AhR anti-body AhR (1:500; Enzo Life Sciences Inc., Farmingdale, NY, USA) and another was probed with IgG control. The brief processes were performed as following IHC methods.

Patients and samples

Fresh tissues of breast cancer compared with their adjacent normal ones were obtained from 10 patients who underwent surgery at Shanghai First Maternity and Infant Hospital from January 2012 to August 2013 (Shanghai, China). Written informed consent to participate in the study was obtained from each patient before surgery, according to the ethical guidelines of Shanghai First Maternity and Infant Hospital. None of the patients has received any preoperative treatment. Tumor phenotype and immunohistochemical characterization were evaluated and defined by pathology department of the hospital. All the 10 pared tissues were tested for AhR and p53 expression by western blot respectively.

Immunohistochemistry

Immunolocalization of AhR was performed using the human breast cancer tissue microarray as described. The sections were incubated in a 10 mM citrate buffer solution (pH 6.0) in a microwave for 10 min for antigen retrieval. Endogenous peroxidase activity was quenched by immersing the tissue sections in 3% H_2O_2 in methanol for 10 min. Sections were incubated with blocking solution and primary antibody, then incubated with biotin-labeled secondary antibody for 10 min. A diaminobenzidine (DAB) kit (Sigma Diagnostics, St Louis, MO) was used as substrate chromogen. Sections were counterstained in Mayer's hematoxylin. The intensity of immunohistochemical reactions was estimated independently by two pathologists. In doubtful cases a reevaluation was performed using a double-headed microscope and staining was discussed until a consensus was achieved. Thus in order to evaluate the AhR expression, immunoreactive Remmele score (IRS) by Remmele and Stegner were applied (**Table 1**) [14]. In IRS scale the intensity of color reaction and percentage of positive cells were taken into account (**Table 2**). The score represented a product of points given for the evaluated characters and it ranged from 0 to 12. For

Table 2. The intensity of color reaction and percentage of positive cells in IRS scale

Definition	Score
No reactivity or membranous reaction in <10% of cells	0
Faint complete or partial membranous reactivity in >10% of cells	1+
Moderate complete or basolateral membranous reactivity in >10% of cells	2+
Strong complete or basolateral membranous reactivity in >10% of cells	3+

the purpose of statistical evaluation, tissues having a final staining score of 1-6 were included in the low AhR expression group and those with scores of 7-12 in the high AhR expression group.

Western blot analysis

Tissues from breast cancers and adjacent normal tissues were lysed and centrifuged at 12,000 rpm for 30 minutes to remove cellular debris. Fifty micrograms of protein was loaded per lane for SDS-PAGE and then transferred onto a PVDF membrane (Millipore, Billerica, MA). After blocking with 5% non-fat milk containing 0.5% Tween 20 for 1 hour at room temperature, the membrane was incubated with anti-AhR (1:2000, Enzo Life Sciences Inc.) or anti-p53 (Cell Signaling Technology, Danvers, MA) antibodies overnight at 4°C. The membrane was then incubated with secondary antibody (1:3000) for 1 hour at room temperature. Proteins on the membrane were visualized using electrochemiluminescence (ECL, Perkin Elmer, Waltham, MA) reagents. The immunoreactive signals were analyzed by densitometry. Data on AhR were normalized to GAPDH.

Statistics

Data were analyzed using SPSS version 17.0 software. Statistical analysis was performed by one-way ANOVA test followed by Tukey post hoc for comparison of continuous data, and Fisher's exact test and Chi-square for categorical data. The student's t-test was performed to compare protein expression of AhR or p53 in normal and cancer tissues. $P < 0.05$ was considered statistically significant.

Results

AhR is highly expressed in breast cancer tissues

To determine the potential role of AhR in breast cancer, 220 breast cancer tissues and 8 non-cancer tissue samples were collected and characterized for the relative protein expression levels of AhR. The immunohistochemistry analysis indicated that the AhR immunoreactivity was mostly present in breast cancer tissues, but not non-cancer tissues (**Figure 1A**). No positive staining was observed in the preimmune rabbit IgG (data not shown). The semi-quantification analysis revealed that the AhR staining score in the breast cancer tissues were much higher than that in the non-cancer tissues (**Figure 1B**).

Correlations between AhR expression and clinicopathological features

The levels of AhR expression were divided into high group and low group according to the relative expression scores. The relationship of AhR expression to age, histology, grade, TNM, molecular subtypes were determined (**Table 3**). Spearman correlations revealed that AhR expression are tightly correlated with p53 status ($P < 0.05$). Furthermore, Student's t-test found that p53 negative subjects (n = 69) expressed higher levels of AhR than p53 positive subjects (n = 151) (**Figure 2**). In addition, high AhR expression in breast cancer appeared to be significantly associated with different histological type. High AhR expression was frequently observed in the invasive ductal breast cancer, but not invasive lobular cancer and other types of breast cancer ($p = 0.0296$). No statistical significances were examined between AhR expression and other variables.

AhR and p53 expression in breast cancer and adjacent normal tissues

Samples from the 10 patients that contained both cancerous and matched noncancerous tissues were evaluated for AhR and p53 protein expression by Western blot. As shown in **Figure 3**, AhR protein levels in breast cancer tissues were 2.6 fold higher (n = 10, $P < 0.05$) than those in normal tissue, however p53 protein expression was significantly reduced in cancer tissues in comparison with normal tissues. The results were well consistent with the results of immunohistochemistry that p53 positive breast cancer tissues contained low levels of AhR protein.

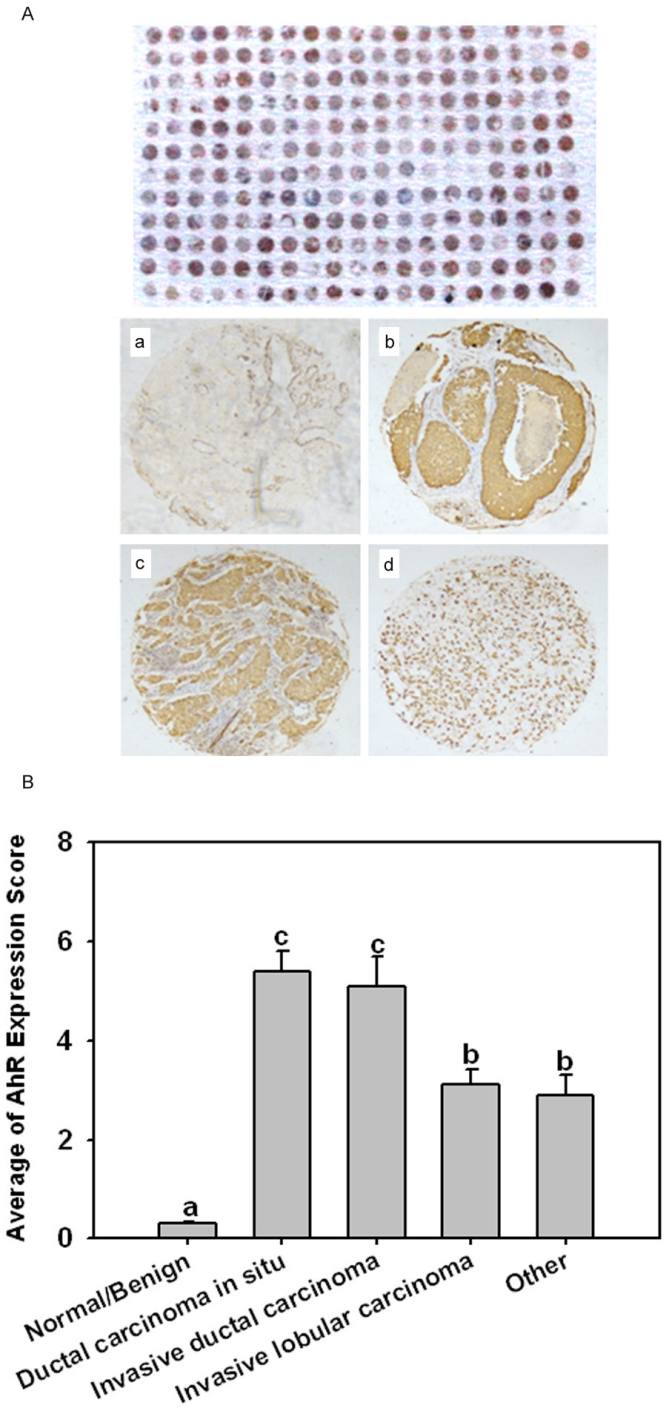

Figure 1. Immunohistochemical analysis of AhR in human breast cancer tissues. Brown color indicates positive AhR staining. (A) Representative images from tissue array (top), Normal (a), Ductal carcinoma in situ (b), Invasive ductal carcinoma (c), Invasive lobular carcinoma (d) are shown. Bar, 100 mm. (B) Semi-quantitative analysis for AhR staining intensities for Norm/Benign (n = 8), Ductal carcinoma in situ (n = 17), Invasive ductal carcinoma (n = 178), Invasive lobular carcinoma (n = 13), others (n = 12). Semi-quantitative data are expressed as Means ± SEM fold of the expression score. *Difference from Normal ($P < 0.05$).

Discussion

In this study, we found that the relative levels of AhR expression in breast cancer tissues were significantly higher than that in the non-cancer tissues. The AhR was predominantly expressed in the cytoplasm of breast tissue cells and high levels of AhR expression were detected in 94 (42.7%) of 220 patients with breast cancer. Furthermore, we found that the high AhR expression was associated significantly with P53 expression ($P = 0.0272$) and histological type ($P = 0.0296$) in patients with breast cancer. To the best of our knowledge, our findings provided the first evidence to demonstrate high levels of AhR protein expression in breast cancer tissues and suggest that high levels of AhR expression may be valuable for the diagnosis of patients with breast cancer as well as a potential therapeutic target for intervention of breast cancer in the clinic.

Breast tumorigenesis is a multi-step process starting from benign and atypical hyperproliferation, progressing into in situ carcinoma, invasive carcinomas, and culminating in metastatic disease. The development and progression of breast cancer are involved in a complex process and attributed to the interaction of many genetic, epigenetic and environmental factors [15]. Altered AhR gene expression profiles may drive the disease progression [16]. The results presented in this study demonstrated that AhR expression was significantly up-regulated in breast cancer tissues, suggested that AhR might function as a complex gene battery in the tumorigenesis of breast cancer. These findings are consistent with AhR expression observed for other types of cancer such as gastric cancer, non-small cell lung cancer, and mammary gland tumors [17-19]. Furthermore, analysis of AhR in breast cancer patients demonstrated that AhR expression was associated with breast pathological type and p53 sta-

Table 3. Correlation of AhR expression with clinical characteristics of breast cancers. The semi-quantitative analysis revealed that for each target protein examined, there was no significant Difference in staining intensity between grades, TNM, ER, PR, C-erbB2, Ki-67, AR, EGFR, Molecular subtype, except for P53 and histological types

Variables			AhR expression		*P* value
		Total n=220	High n = 94	Low n = 126	
Age	≤50	127	56	71	
	>50	93	38	55	0.6319
Histology	Cancer in situ	17	7	10	
	Invasive ductal cancer	178	83	95	
	Invasive lobular cancer	13	3	10	
	Other	12	1	11	0.0296
Grade	I	15	7	8	
	II	176	70	106	
	III	22	13	9	0.2102
TNM	0	17	7	10	
	I	21	6	15	
	II	145	65	80	
	III	37	16	21	0.5720
ER status	Positive	119	52	67	
	Negative	101	42	59	0.7522
PR	Positive	94	39	55	
	Negative	126	55	71	0.7485
HER2 status	Positive	149	59	90	
	Negative	71	35	71	0.2832
Ki-67 status	Positive	102	42	60	
	Negative	118	52	66	0.6655
AR	Positive	118	48	70	
	Negative	102	46	56	0.5087
P53	Positive	151	57	94	
	Negative	69	37	32	0.0272
EGFR	Positive	36	16	20	
	Negative	184	79	105	0.8672
Molecular subtype	Luminal A	40	52	67	
	Luminal B	94	42	59	
	Her2-like	52	39	55	
	Basal-like	34	55	71	0.9772

tus, but not patients' age, tumor grade and TNM. Although AhR expression was significantly correlated with histological grade, pathological T stage, lymphovascular invasion and lymph node involvement in urinary tract urothelial carcinoma [20]. These findings implicated that AhR might play an important role in the multiple stages of tumor development.

The importance of some molecular markers in breast cancer has been of considerable interest during recent years, not only as prognostic markers, but also as predictors of response to therapy. The p53 protein, a prototypical tumor suppressor, plays a central role in the growth and differentiation of normal breast epithelium [21]. In its normal form, p53 can be involved in the induction of apoptosis and thus has a regulatory function over the cell cycle [22]. Herein, we found p53 positive breast cancers contained low levels of AhR protein, suggested a negative correlation between p53 protein lev-

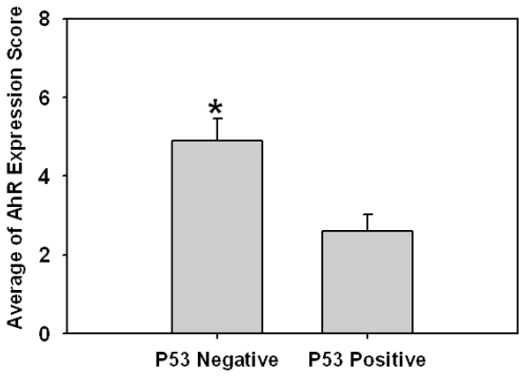

Figure 2. AhR expressions in human breast cancers with different status of p53. Semi-quantitative data are expressed as Means ± SEM of the expression score. *Differ from Normal ($P < 0.05$).

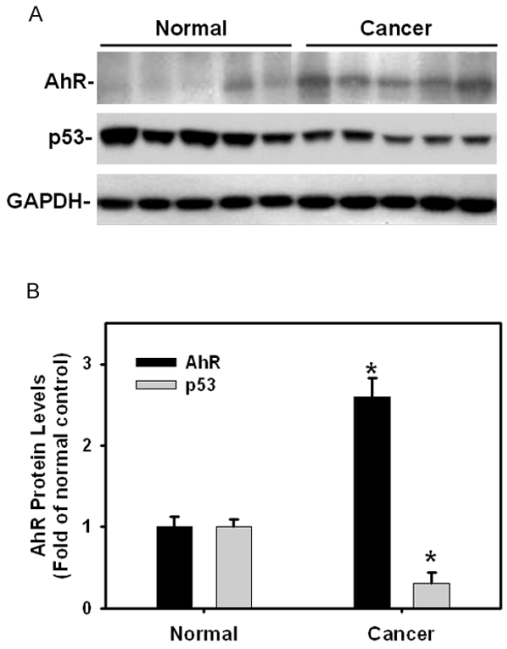

Figure 3. AhR and p53 protein expressions in human breast cancer (cancer) and corresponding adjacent normal tissues (Normal). AhR and p53 protein expression was detected by Western blot (A) and band intensities of AhR were normalized with corresponding band intensities of GAPDH (B). *Significantly different from normal controls ($P < 0.05$).

els and AhR expression. Experiments in vitro and animal have also shown a close interaction between AhR and p53 [23]. In vitro studies suggested that inhibition of p53 by its specific inhibitor induced AhR activation in a rainbow trout and rat hepatoma cell lines. Similarly, silencing or overexpression of wild-type p53 enhanced and suppressed CYP1A1 and CYP1B1 expression, respectively, suggested a negative relation between p53 and AhR activity [24]. Meanwhile, AhR ligand TCDD can counteract the activity of p53 (phosphorylation and acetylation) triggered by genotoxicants in the human hepatocarcinoma cell line HepG2 [25]. In addition, another study reported that simultaneous exposure to TCDD resulted in a inhibition of p53 expression [26]. These results suggested that AhR ligands might contribute to tumor progression by inhibiting p53 expression and activation (phos-phosphorylation and acetylation). Based on these studies, further investigation about the relation between AhR and p53 appear to be warranted in breast carcinogenesis.

In conclusion, our data from this study showed that high levels of AhR were expressed in the majority of breast cancer tissues and closely associated with P53 status as well as histological types of breast cancer. As suggested in many works, AhR may be a potential novel drug-interfering target for cancer, and further understanding the function and molecular mechanisms of AhR in regulating the progression of breast cancer may provide new insights into breast tumorigenesis.

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Disclosure of conflict of interest

None.

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