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Prognostic role of Amphiregulin and the correlation with androgen receptor in invasive breast cancer

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ABSTRACT

Background: In androgen-sensitive prostate cancer, androgenic stimulation induces the synthesis of amphiregulin (AREG). Research is lacking on the role of AREG in invasive breast cancer and the co-expression with androgen receptor (AR) status.

Materials and methods: The present study investigated the prognostic role of AREG in invasive breast cancer cases ($N = 298$) and the co-expression with the AR status as analysed by immunohistochemistry (IHC).

Results: The samples were divided into groups according to AREG expression levels: low/no expression ($AREG^{low/no}$) and high expression ($AREG^{high}$). As shown by cytoplasmic immunostaining, 46.0% (137/298) of invasive breast cancers were $AREG^{high}$, and 54.0% (161/298) of cases were $AREG^{low/no}$. Co-expression of the AR and AREG accounted for 62.4% (186/298) of cases. A Kaplan–Meier analysis revealed that $AREG^{high}$ and AR^{+}/$AREG^{high}$ decreased patients' overall survival (OS) ($P = 0.002$ and $P = 0.006$, respectively) and disease-free survival (DFS) ($P < 0.001$ and $P < 0.001$, respectively). In Cox models, AR^{+}/$AREG^{high}$ remained an independent prognostic indicator of OS and DFS in invasive breast cancer (hazard ratio [HR], 0.591, 95% confidence interval [CI], 0.407-0.859, $P = 0.006$; HR, 0.449, 95% CI, 0.236-0.853, $P = 0.014$, respectively). $AREG^{high}$ remained an independent prognostic indicator of OS and DFS in estrogen receptor (ER)-negative tumours ($P < 0.05$).

Conclusions: This study suggested that AREG and the AR were co-expressed in invasive breast cancer. Thus, AREG and the AR may be valuable prognostic biomarkers in invasive breast cancer and promising therapeutic targets, especially in ER-negative breast cancer.

1. Introduction

Breast cancer is a global health issue due to its high incidence rate [1]. Local recurrence and distant metastasis, as well as therapeutic resistance, are major treatment challenges at present [2,3]. There is an urgent need for the identification of effective biomarkers to monitor and predict the prognosis in breast cancer.

Shoyab et al. [4] was the first to purify human amphiregulin (AREG) from conditioned medium of an estrogen receptor (ER)-positive breast cancer cell line, MCF-7, after treatment with phorbol 12-myristate 13-acetate. Physiologically, AREG plays an important role in the development and maturation of adolescent mammary glands and in the formation of terminal ductal lobular units [5]. In the absence of AREG, mammary ductal growth is stunted and fails to expand [6]. Pathologically, AREG functions as a pro-oncogenic molecule through autocrine and paracrine pathways, with elevated AREG expression implicated in

Abbreviations: AREG, amphiregulin; AR, androgen receptor; ER, estrogen receptor; PR, progesterone receptor; TNBC, triple-negative breast cancer; HER2, human epidermal growth factor receptor 2; IHC, immunohistochemistry; ASCO-CAP, American Society of Clinical Oncology and the College of American Pathologists; FISH, fluorescence in situ hybridization; OS, overall survival; DFS, disease-free survival; IDC-NOS, invasive ductal cancers not otherwise specified; HR, hazard ratio; CI, confidence interval

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various disorders, including the initiation and progression of malignancies [7]. AREG also acts as a natural ligand for the epidermal growth factor receptor (EGFR) in the activation of growth-signalling pathways via a downstream signal cascade [8]. A previous study on the function of AREG in vivo in *Areg* gene knockout mice reported impaired mammary gland maturation, leading to difficulty in offspring feeding [9]. ER-positive cells secrete AREG, which then contributes to the regulation of self-renewal and differentiation potential of neighbouring ER-negative stem cells [10]. However, the prognostic role of AREG in invasive breast cancer, including ER-negative breast cancer, is unclear.

The androgen receptor (AR), a prevalent sex steroid hormone receptor that functions in a wide range of biological processes in breast cancer, is expressed in 60%–70% of breast cancers [11–14]. In some cases, it is more highly expressed than the ER or progesterone receptor (PR) [13,15,16]. Emerging experimental and clinical data have indicated that the AR is an attractive target in breast cancer treatment [14,17]. In androgen-sensitive prostate cancer, AREG expression was detected at both transcriptional and translational levels, and AREG expression was up-regulated by androgenic stimulation [18]. Barton et al. [19] reported that AREG expression was associated with both up- and down-regulation of AR in AR-positive triple-negative breast cancer (TNBC). There is a lack of research on the oncogenic function of AREG and the AR in human breast cancer. In this study, the immunohistochemical expression of AREG and AR and the prognostic values of AREG and AR/AREG co-expression were investigated in human invasive breast cancer, particularly ER-negative breast cancer.

2. Materials and methods

2.1. Specimens' parameters

This retrospective cohort study randomly recruited ER-positive ($n = 149$) and ER-negative ($n = 149$) female breast cancer patients from the Cancer Hospital of Tianjin Medical University between March 17, 2008 and November 18, 2011. The inclusion criteria were as follows: primary invasive breast cancer, no neoadjuvant therapy before surgery and with complete medical records. Data on each patient's age, menopausal status and the pathological tumour stage were retrospectively obtained by reviewing the medical records. In addition, haematoxylin and eosin slides were reviewed again for confirmation of the diagnosis, histological grade and lymph node metastasis status. Data on the status of the ER, PR, human epidermal growth factor receptor 2 (HER2), Ki-67 and p53 were based on a review of IHC-stained sections. All clinicopathological parameters were available. Patients were treated following the National Comprehensive Cancer Network (NCCN) breast cancer treatment guidelines [20]. Moreover, four fresh breast cancer tissues and adjacent non-tumoural breast tissues were obtained for Western blotting analysis, and their formalin-fixed and paraffin-embedded materials were used for immunohistochemistry (IHC) of AREG.

ER and PR was considered positive in cases of ≥1% nuclear stained cells [21]. Tumours were considered positive for HER2 if IHC was scored as 3+ and tumours with HER2 scored as 2+ were further confirmed through fluorescent in situ hybridization (FISH) test [22]. Samples with more than 20% of tumour cells showed strong nuclear Ki-67 staining were scored as Ki-67 high proliferation [23]. The cut-off point for p53 positivity was set at ≥ 10% of tumour cells, with strong nuclear reactivity [24]. As previously described [23], Luminal A subtype: ER and PR positive, HER2 negative and Ki-67 < 20%; Luminal B (HER2 negative) subtype: ER positive, no or low PR expression, HER2 negative and Ki-67 ≥ 20%; Luminal B (HER2 positive) subtype: ER positive, any PR expression, HER2 positive and any Ki-67 index; HER2-positive: the absence of ER and PR expression, HER2 overexpression or gene amplification; TNBC: no expression of ER, PR and HER2.

2.2. IHC analysis and scoring

The protein expression of AREG and the AR were detected by IHC. The specimens (4-μm sections) were fixed in 10% neutral buffered formalin and then paraffin embedded. The sections were then deparaffinized in xylene and rehydrated in an ethanol gradient. Antigen retrieval was carried out in citrate buffer solution for AREG and in ethylene diamine tetraacetic acid buffer for the AR for 2 min 30 s in an autoclave. The specimens were then immersed in 3% hydrogen peroxide for 10 min to block activation of endogenous peroxidase. Subsequently, the sections were blocked with normal goat serum for 30 min and incubated overnight at 4 °C with anti-human AREG antibody (bs-3847R; Biosynthesis Biotechnology, Beijing, China) at 1:100 dilution or anti-human AR antibody (ZA-0554; ZSGB, Beijing, China) at 1:200 dilution. After washing with phosphate-buffered saline, the sections were incubated with biotin-labeled secondary antibody and streptavidin horseradish peroxidase according to the manufacturer's protocol. Visualization was performed with 3, 30-diaminobenzidine as the chromogen-substrate. The slices were then counterstained with haematoxylin. Positive controls included normal prostate tissues for the AR and a known positive external control in breast cancer for AREG. For negative controls, primary antibodies were substituted by normal goat serum. In accordance with previous research [25], the AR was considered positive in cases of ≥10% nuclear stained cells. For AREG expression evaluation, the method of Li et al. [26] was applied. The AREG IHC scores were calculated by multiplying the proportion of positive cells by the staining intensity, according to the percentage of positive cells (1, < 25%; 2, ≥ 25% and < 50%; 3, ≥ 50% and < 75% and 4, ≥75%) and cytoplasmic staining intensity (0, negative; 1, weak; 2, moderate and 3, strong). Based on the results, the following subgroups were defined: scores of 0–6 were defined as low/no expression ($AREG^{low/no}$), and scores 8–12 were considered high expression ($AREG^{high}$).

2.3. Western blot analysis

Each protein sample was separated by 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (Invitrogen, CA, USA) and transferred onto a polyvinylidene difluoride membrane (Millipore, MA, USA). The blots were exposed at 4 °C overnight with specific primary anti-AREG antibodies (ab33558; Abcam, USA), and anti-β-actin (8H10D10; CST, USA) was used as a loading control. Each sample was done in triplicate.

2.4. Follow-up study

After treatment completion, the patients were followed up via telephone every 3 months in the first year, at 6-month intervals in the second year and at 12-month intervals thereafter. The primary endpoint events were overall survival (OS) and disease-free survival (DFS). OS was determined from the date of surgery to the date of death or the follow-up time. DFS was determined from the date of surgery to the date of local relapse or distant metastasis confirmation. Local recurrence was considered as reappearance of cancer in the treated remnant breast, chest wall or skin. Distant metastasis was considered recurrence at any distant site, such as the liver, lung, brain or other organs. The follow-up ended on March 5, 2018. The follow-up time ranged from 77 to 121 months, with a median follow-up duration of almost 96 months.

2.5. Statistical analysis

Statistical analyses were conducted using SPSS 22.0 software (SPSS Inc., Chicago, IL, USA). The association of AREG and the AR alone and AR/AREG co-expression with various clinicopathological factors were examined using the $\chi 2$ test. The correlation between AREG and the hormone receptor was tested using Spearman's rank correlation test.

Kaplan–Meier survival curves were used to assess OS and DFS, both of which were determined using the log-rank test. Multivariate analysis was performed using Cox proportional-hazards regression models. P values < 0.05 were considered statistically significant.

3. Results

3.1. Clinicopathological parameters

The study cohort consisted of 298 female patients with invasive breast cancer. The cancer types were as follows: invasive ductal cancer not otherwise specified (IDC-NOS) (n = 230), IDC mixed with lobular or other types (n = 27), invasive lobular cancer (n = 18), carcinoma with apocrine differentiation (n = 6), invasive papillary carcinomas (n = 5), invasive micropapillary carcinomas (n = 4), carcinoma with neuroendocrine features (n = 4), mucinous cancer (n = 3) and invasive cribriform cancer (n = 1). Supplementary Table 1 presents information on the patients' general clinical characteristics.

3.2. Localization of AREG and AR proteins

Fig. 1A shows the localization of the AREG protein, with strong cytoplasmic staining detected in invasive breast cancer tissues. Immunoreactive AREG was also found in adjacent non-tumoural tissues (Fig. 1B). Western blotting and IHC revealed AREG protein expression in four paired tumours and adjacent non-tumoural breast tissues (Supplementary Fig. 1A and B). These results confirmed that AREG was commonly expressed in tumours but at a lower level in adjacent non-tumoural breast tissues. The AREG protein was not expressed in stromal or vascular tissues or inflammatory cells. As shown by the AR protein pattern of staining, it was predominantly localized in the nucleus of breast tumour cells, with strong staining (Fig. 1C). In a few cases, the protein was localized in both the nucleus and cytoplasm (Fig. 1D).

3.3. Correlations of AREG and AR expression alone and AR/AREG co-expression with clinicopathological characteristics

Among the 298 samples, 46.0% (137/298) of the samples were classified as $AREG^{high}$, and 54.0% (161/298) of cases were classified as $AREG^{low/no}$. $AREG^{high}$ was associated with the Ki-67 index (P < 0.001), pathological tumour stage (P = 0.009), EGFR status (P < 0.001) and tumour type (P = 0.037). The presence of $AREG^{high}$ tended to be associated with lymph node involvement (P = 0.002). The frequency of tumours expressing AREG varied across the molecular subtypes (P = 0.001). Immunohistochemically, AREG expression was most prevalent in the luminal A subtype (55.5%, 61/110), followed by the HER2-positive subtype (52.0%, 39/75), luminal B subtype (43.6%, 17/39) and TNBC subtype (27.0%, 20/74). Differences were found between AREG expression with the ER status (P = 0.027) and PR status (P = 0.021). Overall, 67.4% (201/298) of cases displayed nuclear immunoreactivity for the AR protein. AR-positive expression was statistically significantly associated with the ER, PR, Ki-67 index and molecular subtypes (P < 0.001, P < 0.001, P < 0.001 and P < 0.001, respectively). Of note, $AR^{+}/AREG^{high}$ was observed in 37.9% (113/298) of patients. $AR^{+}/AREG^{high}$ was more common in lymph node positive cases (45/89, 50.6%) than in lymph node negative cases (68/209, 32.5%; P = 0.017). $AR^{+}/AREG^{high}$ was associated with the ER, PR, EGFR status and molecular subtypes (P < 0.05) (Table 1; Fig. 2).

The results of Spearman's rank correlation analysis of the correlation between AREG expression and the hormone receptors other than the ER, PR (i.e. AR) revealed that AREG protein expression was positively correlated with the AR status (r = 0.296, P < 0.001). More importantly, $AREG^{high}$ expression was detected in 56.2% (113/201) of samples in the AR-positive group as compared with 24.7% (24/97) of samples in the AR-negative subgroup. $AREG^{low/no}$ expression was detected in 75.3% (73/97) of samples in the AR-negative group as compared with 43.8% (88/201) of samples in the AR-positive subgroup. Thus, co-expression of AR and AREG accounted for 62.4% (186/298) of cases (Table 2).

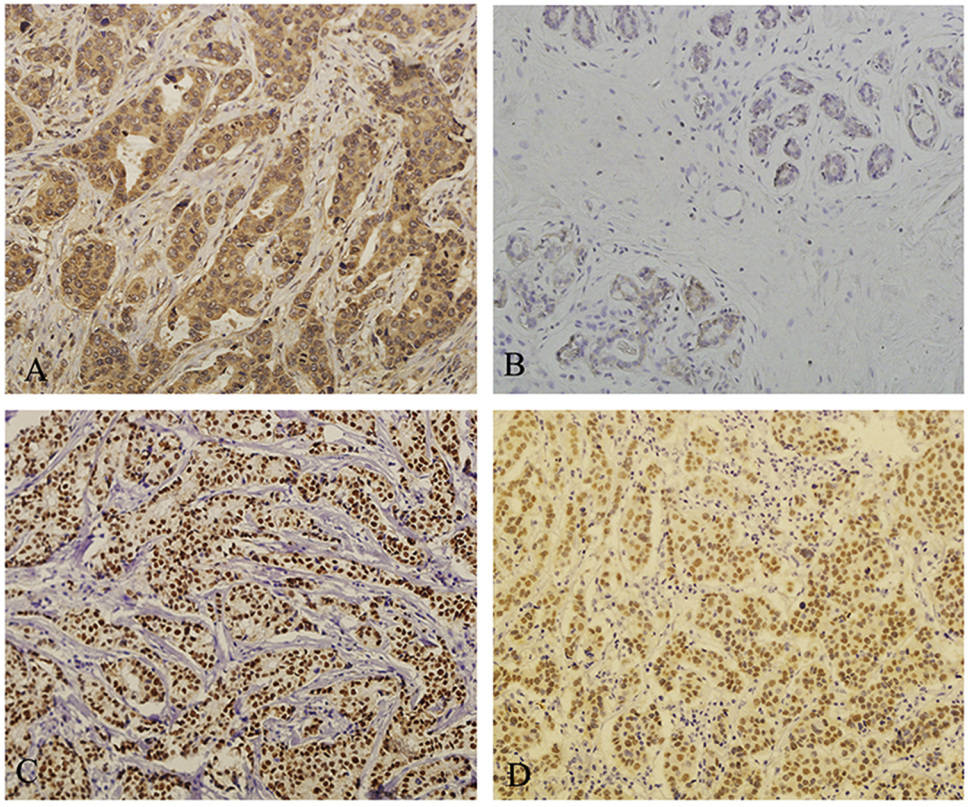

Fig. 1. Immunohistochemical staining of AREG and AR protein expression. Immunohistochemical staining of AREG detected in cytoplasmic staining in invasive breast cancer tissues (A) and in adjacent non-tumoural tissues (B). Immunohistochemical staining of AR revealed nuclear staining (C) and both nuclear and cytoplasmic staining (D) in invasive breast cancer tissues. (original magnification ×200).

Table 1
Correlations of AREG and AR expression alone and AR/AREG co-expression with clinicopathological factors in invasive breast cancer.

Factors	Total	$AREG^{high}$ No. (%)	$\chi2$	*P* value	AR-positive No. (%)	$\chi2$	*P* value	$AR^+/AREG^{high}$ No. (%)	$\chi2$	*P* value
	298	137(46.0)			201(67.4)			113(37.9)		
Age (years)			5.428	**0.020**[a]		5.095	**0.024**[a]		9.710	**0.021**[a]
≤50	117	44(37.6)			70(59.8)			32(27.4)		
> 50	181	93(51.4)			131(72.4)			81(44.8)		
ER			4.877	**0.027**[a]		18.723	**P < 0.001**[a]		20.580	**P < 0.001**[a]
Negative	149	59(39.6)			83(55.7)			42(28.2)		
Positive	149	78(52.3)			118(79.2)			71(47.7)		
PR			5.311	**0.021**[a]		14.463	**P < 0.001**[a]		16.485	**0.001**[a]
Negative	165	66(40.0)			96(58.2)			49(29.7)		
Positive	133	71(53.4)			105(78.9)			64(48.1)		
HER2			2.164	0.141		2.410	0.121		4.330	0.228
Negative	200	86(43.0)			129(64.5)			68(34.0)		
Positive	98	51(52.0)			72(73.5)			45(45.9)		
p53 overexpression			0.173	0.677		5.658	**0.017**[a]		5.755	0.124
Negative	180	81(45.0)			112(62.2)			64(35.6)		
Positive	118	56(47.5)			89(75.4)			49(41.6)		
Ki-67 index (%)			14.042	**P < 0.001**[a]		16.006	**P < 0.001**[a]		23.219	**P < 0.001**[a]
< 20%	172	95(55.2)			132(76.7)			82(47.6)		
≥20%	126	42(33.3)			69(54.8)			31(24.6)		
EGFR			16.003	**P < 0.001**[a]		0.002	0.968		27.344	**P < 0.001**[a]
Negative	230	91(39.6)			155(67.4)			81(35.2)		
Positive	68	46(67.6)			46(67.6)			32(47.1)		
Lymph node			9.418	**0.002**[a]		0.644	0.422		10.153	**0.017**[a]
Negative	209	84(40.2)			138(66.0)			68(32.5)		
Positive	89	53(59.6)			63(70.8)			45(50.6)		
Histological grade			0.044	0.833		5.844	**0.016**[a]		7.121	0.068
G1-G2	159	74(46.5)			117(73.6)			66(41.5)		
G3	139	63(45.3)			84(60.4)			47(33.8)		
Pathological tumour stage			9.364	**0.009**[a]		5.370	0.068		13.144	**0.041**[a]
pT1	70	40(57.1)			49(70.0)			32(45.7)		
pT2	146	70(47.9)			105(71.9)			59(40.5)		
pT3	82	27(32.9)			47(57.3)			22(26.8)		
Molecular subtype			15.862	**0.001**[a]		35.439	**P < 0.001**[a]		46.067	**P < 0.001**[a]
Luminal A	110	61(55.5)			90(81.8)			55(50.0)		
Luminal B	39	17(43.6)			28(71.8)			16(41.0)		
HER2	75	39(52.0)			53(70.7)			34(45.3)		
TNBC	74	20(27.0)			30(40.5)			8(10.8)		
Tumor type			6.598	**0.037**[a]		2.513	0.285		12.708	**0.048**[a]
IDC	230	115(50.0)			160(69.6)			94(40.9)		
Mixed [b]	27	9(33.3)			15(55.6)			9(33.3)		
Other special types[c]	41	13(31.7)			26(63.4)			10(24.4)		

NOTE: *P* values were calculated by $\chi2$ test.
[a] Difference was statistically significant.
[b] IDC mixed with lobular or other types.
[c] Invasive lobular cancers, carcinoma with apocrine differentiation, invasive papillary carcinoma, invasive micropapillary carcinoma, carcinoma with neuroendocrine features, mucinous cancer and invasive cribriform cancer.

3.4. Prognostic value of AREG expression and co-expression of AR/AREG

Kaplan–Meier survival curves were used to determine whether AREG or AR/AREG co-expression predicted the clinical prognosis. As illustrated in Fig. 3, the outcomes of the AR-positive patients were poorer than those of the AR-negative patients (OS, $P = 0.015$, Fig. 3A; DFS, $P = 0.006$, Fig. 3B). The outcomes of the $AREG^{high}$ patients were poorer than those of the $AREG^{low/no}$ patients (OS, $P = 0.002$, Fig. 3C; DFS, $P < 0.001$, Fig. 3D). The $AR^+/AREG^{high}$ cases had the worst clinical outcomes (OS, $P = 0.006$, Fig. 3E; DFS, $P < 0.001$, Fig. 3F) as compared with those of the other three subgroups. Given that the biological characteristics of the IDCs and non-IDC cases differed, the prognostic significance of AREG expression, with and without AR expression in IDCs and non-IDCs was analysed separately. AREG expression, with and without AR expression contributed to unfavourable outcomes in IDCs ($P < 0.05$, Supplementary Fig. 2A-D) but not in non-IDCs ($P > 0.05$, Supplementary Fig. 2E-H).

Next, univariate and Cox proportional-hazards regression models were applied to determine which markers acted as independent prognostic factors. Table 3 shows the association of OS and DFS with the clinicopathological variables in the 298 cases. As indicated by the Cox models, $AR^+/AREG^{high}$ remained an independent prognostic factor for survival outcomes (OS, hazard ratio [HR], 0.591, 95% confidence interval [CI], 0.407-0.859, $P = 0.006$; DFS, HR, 0.449, 95% CI, 0.236-0.853, $P = 0.014$, respectively). The parameters, lacking of statistically significant in univariate analysis, were not included in the final Cox proportional-hazards regression models for OS and DFS calculations.

3.5. Correlations and significance of AREG and AR expression alone and AR/AREG co-expression with ER status

Of the 149 ER-negative tumours (Table 4), 39.6% (59/149) were classified as $AREG^{high}$, 55.7% (83/149) were classified as AR positive, and 28.2% (42/149) were classified as $AR^+/AREG^{high}$. $AREG^{high}$ was associated with AR status, EGFR status and HER2 status ($P < 0.05$). $AR^+/AREG^{high}$ tumours tended to be commonly HER2 enriched (81.0%, 34/42). The results of the Kaplan–Meier survival analysis showed that the AR-positive patients had poorer DFS than those of the AR-negative patients ($P = 0.004$, Fig. 4B), but not for OS ($P > 0.05$, Fig. 4A). Both $AREG^{high}$ and $AR^+/AREG^{high}$ tumours were related to a poor survival outcome ($P < 0.05$, Figs. 4C-F). In the Cox models, AREG alone was an independent prognostic factor for OS and DFS ($P < 0.05$, Supplementary Table 2). Given that ER-negative breast cancer belonged to HER2 and TNBC groups, the prognostic significance of AREG

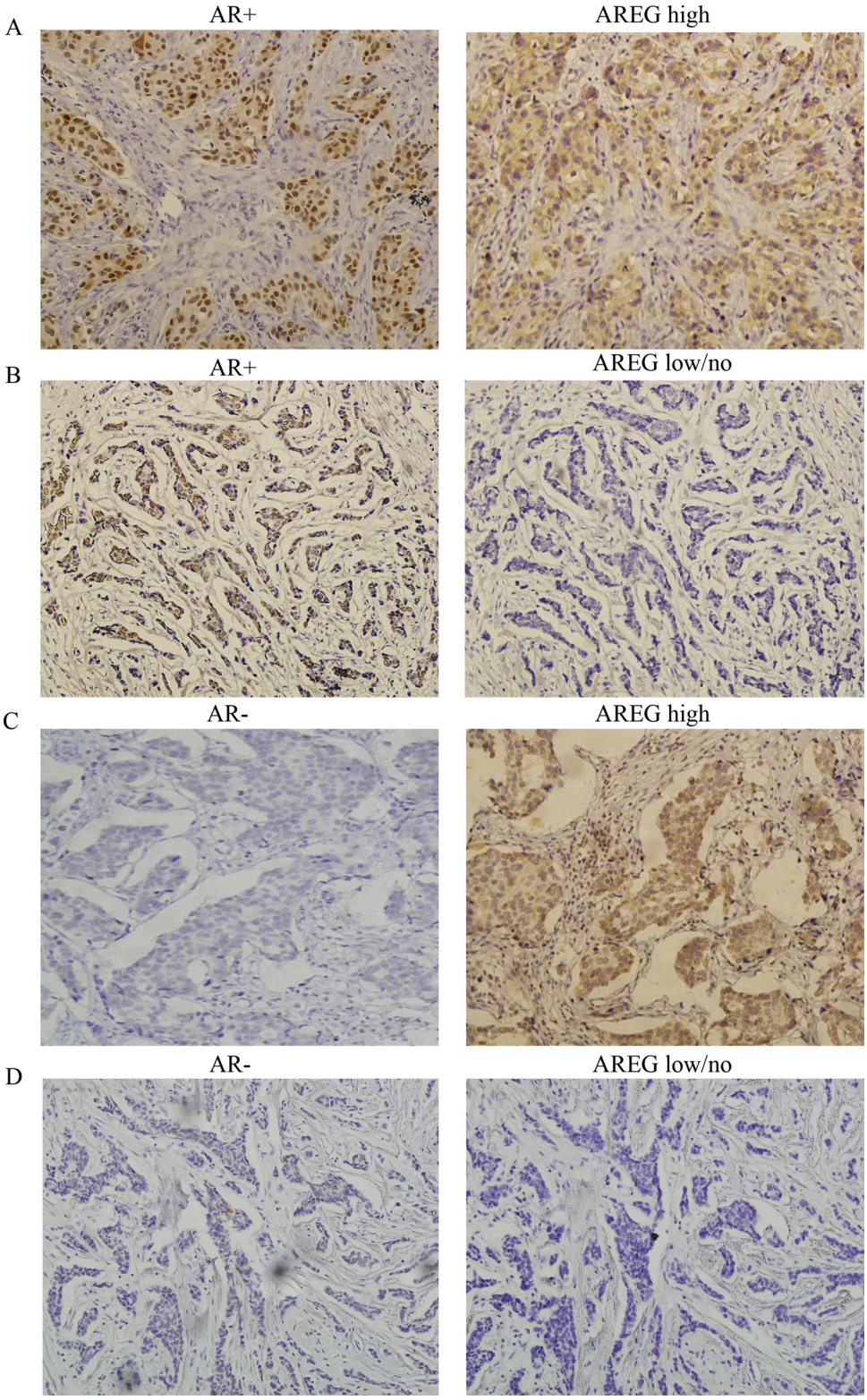

Fig. 2. Continuous sections with immunohistochemical staining of AR and AREG protein expression levels in invasive breast cancer tissues: A. AR-positive nuclear staining and high expression level of AREG in cytoplasm ($AR^+/AREG^{high}$); B. AR-positive nuclear staining and low/no expression level of AREG ($AR^+/AREG^{low/no}$); C. AR-negative-stained and high expression level of AREG in cytoplasm ($AR^-/AREG^{high}$); D. AR-negative-stained and low/no expression level of AREG ($AR^-/AREG^{low/no}$) (original magnification ×200).

expression in these two groups was analysed separately. The results showed that $AREG^{high}$ predicted unfavourable outcomes in HER2-positive breast cancer ($P < 0.05$, Supplementary Figs. 2I-J). In contrast, $AREG^{high}$ was not associated with OS in TNBC ($P > 0.05$, Supplementary Fig. 2K).

Among the 149 ER-positive specimens, $AR^+/AREG^{high}$ was detected in 47.7% (71/149) of cases. $AR^+/AREG^{high}$ was associated with parameters, such as age, menopausal status, p53 status and EGFR status

Table 2
Correlation analysis between AREG and the hormone receptors. **-.**

Hormone receptors	Total (N = 298)	AREG		r	P value
		High % (N = 137)	Low/no % (N = 161)		
AR				0.296	**P < 0.001**[a]
Negative	97	24.7(24)	75.3(73)		
Positive	201	56.2(113)	43.8(88)		
ER				0.128	**0.027**[a]
Negative	149	39.6(59)	60.4(90)		
Positive	149	52.3(78)	47.7(71)		
PR				0.133	**0.021**[a]
Negative	165	40.0(66)	60.0(99)		
Positive	133	53.4(71)	46.6(62)		

NOTE: P values were calculated by Spearman rank correlation test.
[a] Difference was statistically significant.

($P < 0.05$, Supplementary Table 3). Overall, AREG expression alone or co-expressed with the AR was not associated with the clinical prognosis of the patients ($P > 0.05$, Supplementary Fig. 2M-P).

4. Discussion

Although the oncogenic protein AREG is overexpressed only in the cytoplasm in invasive breast cancer, the localization of the AREG protein varied in other cancers; for instance, AREG was localized in both the nucleus and cytoplasm in colon neoplasms [27] and in the nucleus of malignant ovarian epithelial cells [28]. The pattern of AR staining in breast tumour cells was mostly nuclear, and nuclear and cytoplasmic staining have also been observed. Previous research attributed this staining pattern to the localization of the AR [29,30]. When localized in the nucleus, it functions as a transcription factor to mediate the proliferation and differentiation of breast cancer cells [29]. In contrast, when localized in cytoplasm, the AR protein stimulates proliferation via

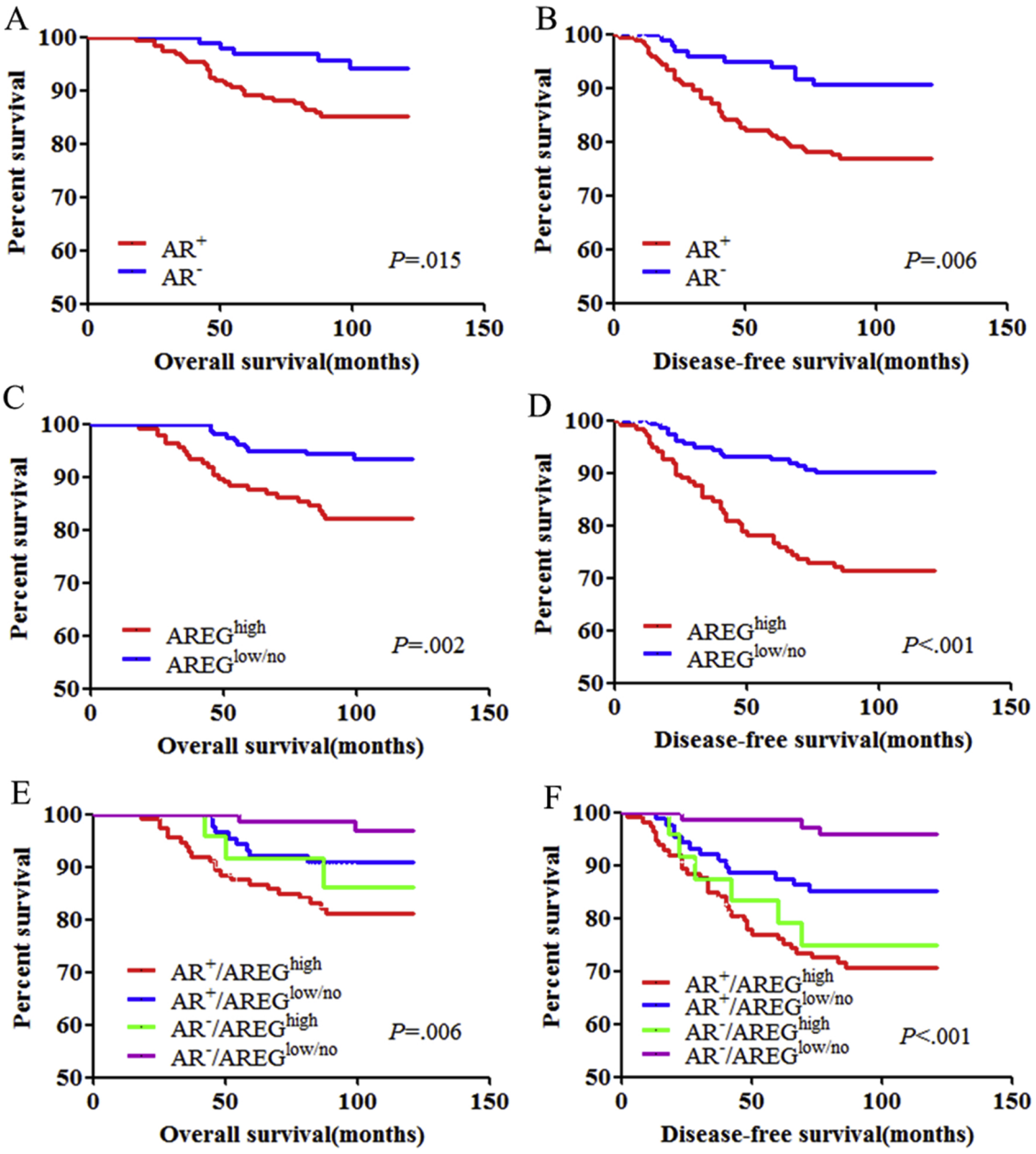

Fig. 3. Kaplan–Meier survival curves. Overall survival (A) and disease-free survival (B) for AR protein in invasive breast cancer. Overall survival (C) and disease-free survival (D) for AREG protein level in invasive breast cancer. Overall survival (E) and disease-free survival (F) for AR/AREG co-expression in invasive breast cancer.

Table 3
Univariate and multivariate analysis of patients' survival.

Factors	OS			DFS		
	Univariate	Multivariate		Univariate	Multivariate	
	P value	HR(95%CI)	*P* value	*P* value	HR(95%CI)	*P* value
AR	0.015[a]			0.005[a]		
AREG	0.002[a]			P < 0.001[a]		
$AR^+/AREG^{high}$	0.006[a]	0.591(0.407-0.859)	0.006[a]	P < 0.001[a]	0.449(0.236-0.853)	0.014[a]
ER	0.951			0.032[a]	0.508(0.268-0.960)	0.037[a]
PR	0.978			0.059		
HER2	0.032[a]			0.037[a]		
Ki-67 index (%)	0.568			0.608		
p53 overexpression	0.174			0.055		
EGFR	0.017[a]			0.001[a]		
Lymph node	0.001[a]	0.395(0.200-0.780)	0.007[a]	P < 0.001[a]	0.489(0.282-0.850)	0.011[a]
Histological grade	0.074			0.807		
Pathological tumour stage	0.028[a]			0.139		
Tumor type	0.660			0.304		
Age (years)	0.045[a]			0.004[a]		
Menopausal status	0.262			0.020[a]		
Molecular subtype	0.890			0.107		

NOTE: Univariate analysis was evaluated using the log-rank test. Multivariate analysis was evaluated by the Cox proportional-hazards regression models.
HR: hazard ratio; CI: confidence interval.
[a] Difference was statistically significant.

non-genomic mechanisms [30].

In the present study, AREG was highly expressed immunochemically in 46.0% of invasive breast cancer cases, which was slightly higher than that reported by Lejeune et al. [31]. The discord may be attributed to differences in the sample sizes, statistical methods and racial/ethnic groups in the two studies. In addition, AREG was expressed more frequently in tumour tissues than in adjacent non-tumoural breast tissues in the present study, which was similar to that reported previously [32]. This finding may be linked to the important role of AREG in mammary epithelial development [33]. Other research reported that AREG

Table 4
Correlations of AREG and AR expression alone and AR/AREG co-expression with clinicopathological factors in ER-negative tumours.

	Total	ER-negative tumours								
Factors		$AREG^{high}$ No. (%)	χ2	*P* value	AR-positive No. (%)	χ2	*P* value	$AR^+/AREG^{high}$ No. (%)	χ2	*P* value
	149	59(39.6)			83(55.7)			42(28.2)		
Age (years)			2.603	0.107		5.810	**0.016**[a]		6.959	0.073
≤50	52	16(30.8)			22(42.3)			9(17.3)		
> 50	97	43(44.3)			61(62.9)			33(34.0)		
HER2			9.712	**0.002**[a]		13.701	**P < 0.001**[a]		23.783	**P < 0.001**[a]
Negative	74	20(27.0)			30(40.5)			8(10.8)		
Positive	75	39(52.0)			53(70.7)			34(45.3)		
p53 overexpression			0.027	0.870		2.607	0.106		2.907	0.406
Negative	77	30(39.0)			38(49.4)			19(24.7)		
Positive	72	29(40.3)			45(62.5)			23(31.9)		
Ki-67 index (%)			5.534	**0.019**[a]		2.728	0.099		6.837	0.077
< 20%	49	26(53.1)			32(65.3)			20(40.8)		
≥20%	100	33(33.0)			51(51.0)			22(22.0)		
EGFR			22.864	**P < 0.001**[a]		3.908	**0.048**[a]		24.596	**P < 0.001**[a]
Negative	93	23(24.7)			46(49.5)			17(18.3)		
Positive	56	36(64.3)			37(66.1)			25(44.6)		
Lymph node			14.026	**P < 0.001**[a]		0.417	0.519		18.274	**P < 0.001**[a]
Negative	102	30(29.4)			55(53.9)			19(18.6)		
Positive	47	29(61.7)			28(59.6)			23(48.9)		
Histological grade			0.097	0.755		2.875	0.090		3.648	0.302
G1–G2	68	26(38.2)			43(63.2)			21(30.9)		
G3	81	33(40.7)			40(49.4)			21(25.9)		
Pathological tumour stage			4.051	0.132		2.265	0.322		8.702	0.191
pT1	32	15(46.9)			17(53.1)			9(28.1)		
pT2	73	32(43.8)			45(61.6)			23(31.5)		
pT3	44	12(27.3)			21(47.7)			10(22.7)		
AR			9.488	**0.002**[a]						
Negative	66	17(25.8)								
Positive	83	42(50.6)								
AREG						9.488	**0.002**[a]			
Low/no expression	90				41(45.6)					
High expression	59				42(71.2)					

NOTE: *P* values were calculated by χ2 test.
[a] Difference was statistically significant.

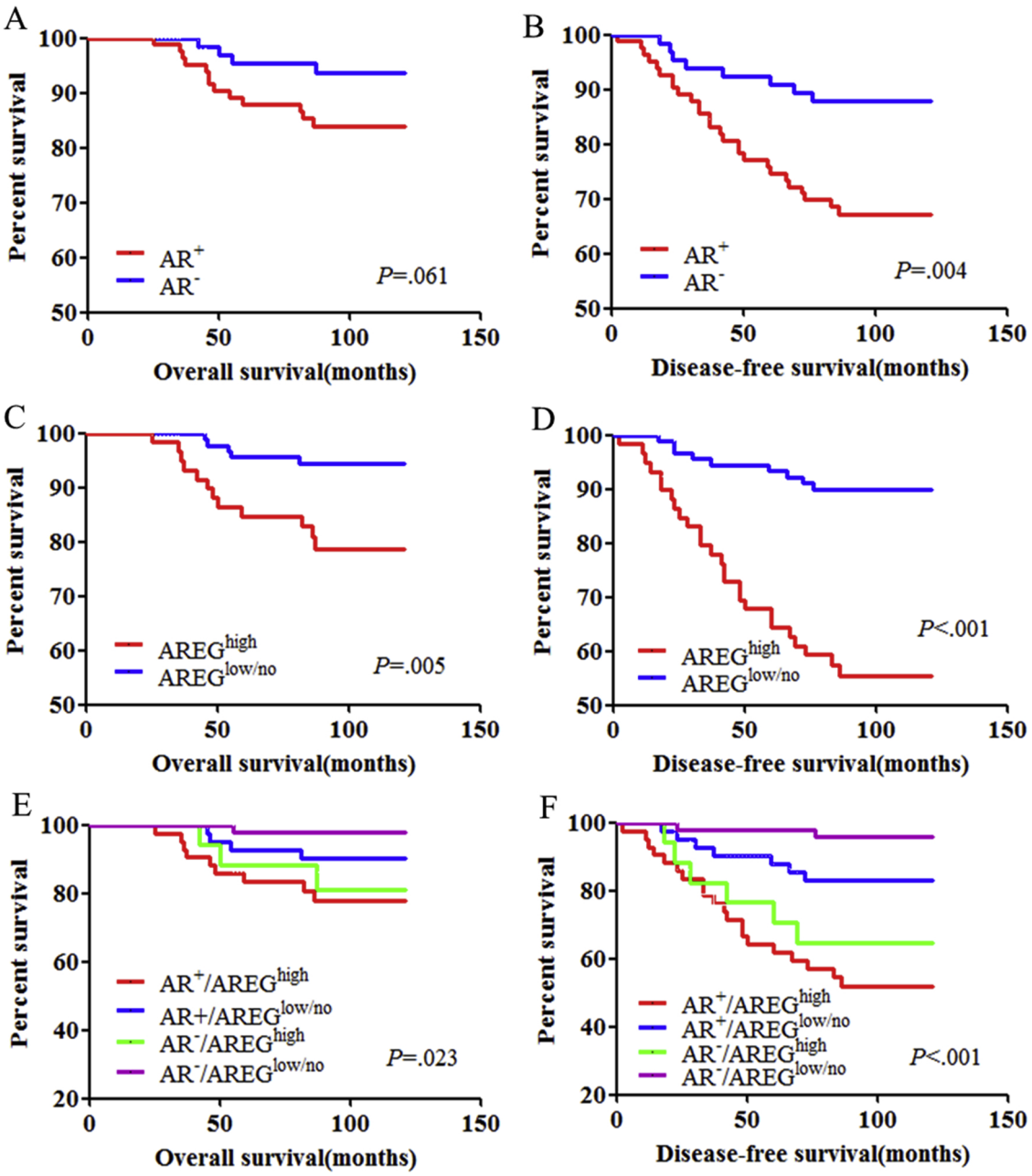

Fig. 4. Kaplan–Meier survival curves. Overall survival (A) and disease-free survival (B) for AR protein in ER-negative breast cancer. Overall survival (C) and disease-free survival (D) for AREG protein level in ER-negative breast cancer. Overall survival (E) and disease-free survival (F) for AR/AREG co-expression in ER-negative breast cancer.

exhibited oncogenic activity in many human epithelial malignancies, including lung, breast and prostate cancer [34].

The analysis of the expression levels of AREG in invasive breast tissues revealed $AREG^{high}$ expression in more than half of AR-positive cases, as well as co-expression between AREG and the AR. In a previous study on human prostate cancer, AREG expression was upregulated via androgenic stimulation [18]. Furthermore, AREG was reported to be a key factor for maintaining androgen-stimulated tumour growth [18]. Barton et al. [19] analysed signalling cross-talk between the AR and AREG and found that the actions of the AR regulated the activity of the AREG signalling pathway.

In the current study, AREG expression appeared to point to an elevated risk of lymph node involvement. Additionally, elevated AREG expression levels were associated with unfavourable outcomes. The multivariate analysis indicated that $AR^{+}/AREG^{high}$ served as an independent marker of the patients' prognoses. These results strongly suggest that AR/AREG co-expression has considerable prognostic relevance in terms of clinical outcomes in invasive breast cancer. In non-IDCs, neither AREG expression nor AR/AREG co-expression had prognostic significance. The limited number of cases may explain this finding. More multicentre studies with large sample sizes are needed to understand the role of AREG in non-IDCs.

According to Shoyab et al. [4], AREG expression was positively correlated with the ER. The findings may be explained by estrogen-induced AREG expression and the role of AREG as a paracrine mediator of the estrogen signalling pathway [35]. Estrogen may increase AREG expression levels in an ER-dependent manner [35]. However, a previous study reported that AREG expression in invasive breast cancer was correlated with the absence of the ER [36]. A possible explanation is that Wicha et al. [37] proposed a potentially significant role for AREG

in ER-negative breast cancer, in which AREG was secreted by ER-positive cells, which then acted to drive the proliferation and differentiation of adjacent ER-negative cells.

To our knowledge, ER-negative breast cancer is not effective against estrogen therapy and is resistant to existing chemotherapy regimens [38], and trastuzumab-targeted therapy is applicable only to some HER2-positive breast cancer patients [39]. As AREG acts as a natural ligand for the EGFR, AREG was associated with the EGFR status in ER-negative and ER-positive breast cancers, and $AREG^{high}$ predicted poor outcomes in HER2-positive breast cancer. These findings were in accordance with Kim et al. [40] who reported that HER2-positive breast cancer patients with high serum AREG levels had significantly shorter progression-free survival and AREG upregulation resulted in increased proliferation of HER2-positive cells in colony-forming assays. In addition, Hurbin et al. [41] demonstrated that AREG inhibited the apoptosis of non-small cell lung cancer cell lines via an IGF1 receptor-dependent pathway, independent of its binding to EGFR. However, whether AREG is independent of EGFR in breast cancer has not been reported.

In conclusion, there was a high frequency of AREG expression in invasive breast tissues, emphasizing the importance of AREG signalling in breast tumour pathogenesis. Furthermore, co-expression of AR/AREG was correlated with an unfavourable prognosis in invasive breast cancer. Results indicate that AREG and the AR may be useful therapeutic targets in breast cancer, particularly in ER-negative breast cancer.

Compliance with ethical standards

The study protocol was approved by the Human Ethical Committee of Tianjin Medical University Cancer Institute and Hospital, and has been performed in consistent with the ethical standards laid down in the 1964 Helsinki Declaration and its later amendments.

Informed consent

Informed consent was obtained from all patients before their surgery and before examination of the specimens.

Author contributions

Yun Niu contributed to design the study and re-evaluate the cases; Guomin Xiang contributed significantly to analysis the results and wrote the manuscript; Fang Liu and Jing Liu performed the data analyses and provided writing assistance; Qingxiang Meng and Nannan Li helped performed the analysis with constructive discussions. All authors read and approved the final manuscript.

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Declarations of interest

none

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.prp.2019.04.006.

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