RESEARCH COMMUNICATION

Retinoid Receptors in Gastric Cancer: Expression and Influence on Prognosis

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Abstract

   Background: Gastric cancer is frequently lethal despite aggressive multimodal therapies, and new treatment approaches are therefore needed. Retinoids are potential candidate drugs: they prevent cell differentiation, proliferation and malignant transformation in gastric cancer cell lines. They interact with nuclear retinoid receptors (the retinoic acid receptors [RARs] and retinoid X receptors [RXRs]), which function as transcription factors, each with three subclasses, α, β and γ. At present, little is known about retinoid expression and influence on prognosis in gastric cancers. Patients and Methods: We retrospectively analyzed the expression of the subtypes RARa, RARβ, RARγ, RXRa, RXRβ, RXRγ by immunohistochemistry in 147 gastric cancers and 51 normal gastric epithelium tissues for whom clinical follow-up data were available and correlated the results with clinical characteristics. In addition, we quantified the expression of retinoid receptor mRNA using real-time PCR (RT-PCR) in another 6 gastric adenocarcinoma and 3 normal gastric tissues. From 2008 to 2010, 80 patients with gastric cancers were enrolled onto therapy with all-trans-retinoic acid (ATRA). Results: RARa, RARβ, RARγand RXRγ positively correlated with each other (p < 0.001) and demonstrated significantly lower levels in the carcinoma tissue sections (p < 0.01), with lower RARβ, RARγ and RXRa expression significantly related to advanced stages (p < =0.01). Tumors with poor histopathologic grade had lower levels of RARa and RARβ in different histological types of gastric carcinoma (p < 0.01). Patients whose tumors exhibited low levels of RARa expression had significantly lower overall survival compared with patients who had higher expression levels of this receptor (p < 0.001, HR=0.42, 95.0% CI 0.24-0.73), and patients undergoing ATRA treatment had significantly longer median survival times (p = 0.007, HR=0.41, 95.0% CI 0.21-0.80). Conclusions: Retinoic acid receptors are frequently expressed in epithelial gastric cancer with a decreased tendency of expression and RARa may be an indicator of a positive prognosis. This study provides a molecular basis for the therapeutic use of retinoids against gastric cancer.

Keywords: Stomach neoplasms - prognostic factors - retinoic acid receptors - ATRA - therapy

Introduction

   The incidence of gastric carcinoma is declining, it remains the second leading cause of cancer-related mortality worldwide (Crew and Neugut, 2006) with five-year survival in France (32.6%), the USA (26%), China (30.5%), Iran (24%) and Japan (screening: 89.4%, no screening: 66.5%) (Msika et al., 2000; Cunningham et al., 2005; Kunisaki et al., 2006; Deng et al., 2010; Khedmat et al., 2011), by the time of diagnosis, most patients have advanced stages of disease (Deng et al., 2010). Its achievement of locoregional control for advanced disease remains very difficult, despite multimodal therapy, 54% of patients had locoregional recurrence (Gunderson and Sosin, 1982). Therefore, ongoing research focuses on the development of more potent therapies. Use of neoadjuvant and adjuvant treatment to further improve results continues

to be investigated. A biological approach might lead to further individualized treatment options.

   Retinoic acid has been recognised as a pivotal compound in cell differentiation, proliferation and malignant transformation (Shyu et al., 1995; Tanaka and De Luca, 2009). Retinoids are natural and synthetic derivatives of retinol (vitamin A). The naturally occuring retinoids, all-trans retinoic acid, 9-cis retinoic acid and 13-cis retinoic acid, are generated from diet-derived retinol. Retinoids are ligands of cellular receptors of retinoic acid, including retinoic acid receptors (RARs) and retinoid X receptors (RXRs) who are members of the steroid and thyroid hormone receptor superfamily (Evans, 1998; Sun and Lotan, 2002). Each subtype of both retinoid receptor classes (RARa, RARβ, RARγ and RXRa, RXRβ, RXRγ respectively) is encoded by separate genes. Multiple isoforms of receptor subtypes exist as a result of

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alternate splicing. The receptors act mainly as RAR–RXR heterodimers, bind to specific DNA sequences (retinoic acid response elements) and act as ligand-dependent transcription factors (Altucci and Gronemeyer, 2001). The RAR family is endogenously activated by ATRA and 9-cis-RA, whereas the RXR family is activated by 9-cis-RA only (Mangelsdorf et al., 1994).

Retinoids are essential in the maintenance of normal adult epithelial differentiation. Vitamin A-deficient experimental animals have long been known to develop squamous metaplasia and other precancerous lesions. These alterations were reversed by vitamin A repletion (Lippman et al., 1994; Sankaranarayanan and Mathew, 1996). Several subsequent studies demonstrated the effect of retinoids in the chemoprevention of certain malignant tumors (Lippman et al., 1994; Sankaranarayanan and Mathew, 1996). The growth inhibitory effect of retinoids has also been extensively studied in established gastric carcinoma cell lines (Shyu et al., 1995; Wu et al., 2000; Xu et al., 2006).

Retinoids strengthen the effect of cytotoxic drugs, such as docetaxel and cisplatin and of ionizing radiationin some cancer cell lines (Jozan and Lafon, 1996; Aebi et al., 1997; Jozan et al., 2002; Scribner Jr and Benbrook, 2002; Wang and Wieder, 2004). The therapeutic effects of retinoids differ from those of traditional cytotoxic agents. Thus, retinoids are potentially fascinating partners for combination therapies with cytotoxic drugs. Certain retinoids are in clinical use, such as all-trans retinoic acid in the treatment of acute promyelocytic leukemia (Sanz et al., 1998) and bexarotene in the therapy of cutaneous T-cell lymphoma (Farol and Hymes, 2004), but their clinical application for the therapy of solid tumors such as gastric cancer is still unknown.

Despite the knowledge of the biological effects of retinoids in gastric cancer, limited information is available on the expression of all isoforms of retinoid receptors in these tumors. Therefore, the objective of the present study was to systematically investigate the expression of RARs and RXRs in paraffin embedded gastric cancer tissues using immunohistochemistry, and to measure the mRNA expression of all RARs and RXRs subtypes in frozen tissue samples with real-time quantitative PCR to evaluated the relation of RARs/RXRs expression with clinicopathological features and also its effect on prognosis, and then, we assessed all-trans retinoic acid was tested for treating the patients with gastric cancer in combination with a cytotoxic agent in vivo.

Materials and Methods

Patients and Tissue samples

147 gastric cancer tissues and 51 distal normal tissues between january 2001 and April 2010 were included in the present study. The surgically resected tissues were fixed in 10% neutral formalin and embedded in paraffin. Haematoxylin and eosin (H&E)-stained tissue sections were screened for histopathologic confirmation based on the WHO Histological Classification (Hamilton and Aaltonen, 2000). All patients were classified according to the 7th edition AJCC/UICC TNM staging systems (Edge et al., 2010). Before processing for RARs/RXRs immunostaining, all available tissue blocks were reviewed for the presence of tumor by a pathologist (LY. C.). Frozen samples for the real-time PCR (RT–PCR) analysis from another 6 gastric adenocarcinoma tissues and 3 normal gastric mucin tissues were collected. Tumor samples were selected by the surgeon and by a a pathologist (H. L.) to exclude macroscopically identifiable non-tumor tissue. The tissue was snap-frozen in liquid nitrogen in the operating theatre and stored at -70 °C until further processing.

Immunohistochemistry

For immunohistochemistry, paraffin-embedded tissue sections were cut (5 um thickness) and mounted on poly-L-lysine-coated slides. The Avidin-Biotin-Peroxidase complex method was described by Hsu (Hsu et al., 1981). Briefly, the sections were dewaxed, hydrated and incubated in 0.5% (v/v) H_2O_2 in methanol for 20 min to block endogenous peroxidase activity. Slides were washed in Tris-buffered saline (TBS) and heated for 5 min at 100°C in 10 mM sodium citrate buffer (pH 6.0) for antigen retrieval. Slides were permitted to cool to 37 °C, followed by rinsing in TBS. Sections were then incubated with a 1:300 dilution of all antibodies overnight at 4 °C in a humidified chamber. anti-RARa (SC-551), anti-RARb (SC-552), anti-RARg (sc-7387), anti-RXRa (sc-553) and anti-RXRg (SC-555) were purchased from Santa Cruz Biotechnology, and anti-RXRb (LS-C38975) antibody from LifeSpan BioSciences. After extensive rinsing with TBS, sections were incubated with biotinylated anti-mouse antiserum and subsequently with horseradish peroxidase streptavidin conjugate. Sections were rinsed and color was developed using 3, 3-diaminobenzidine hydrochloride (DAB) as chromogen. Finally, sections were rinsed in distilled water, counterstained with Mayer's hematoxylin and mounted for evaluation with DPX mountant. The primary antibody was omitted in the negative control. As positive controls, sections of human skin and liver (for all receptors) were incubated with the same antibody. The expression of all antigens defined as nuclear or cytoplasm staining was scored semiquantitatively described by Richter et al., (2002). The immunohistochemical staining was evaluated in 5 areas of the slide sections for correlation and confirmation of the tissue analysis. The results of staining with the percentage and intensity of positive cells for each section was scored 0 to 3 arbitrarily to reflect negative, light, moderate, and strong, respectively. Each slide was evaluated independently by two individuals (LY.C. and H.L.) who both were the Professor of Pathology. Any discrepancy in the scoring of slides was resolved jointly by both of them by discussion and a consensus observation was recorded after discussion.

cDNA synthesis and RT–PCR

Thirty milligrams of frozen tumor tissue was disrupted with a mortar and pestle with concurrent cooling with liquid nitrogen. Total RNA was extracted using the RNeasy Mini kit (Qiagen, Basel, Switzerland) according to the manufacturer's instructions. cDNA was synthesized

Table 1. Real Time PCR Primers Sequences for RARa, RARb, RARg and RXRa, RXRb, RXRg

Gene	Rference of sequence no.	Squence	Product size(bp)	TM	GC%
RARA	NM_000964	F:CTG CTG CCC CTG GAG ATG GAT	217	65.8	61.9
		R: ATG CTT CGC AGG TCA GTA ATC TTC A		64.5	44
RARB	NM_000965	F: CC TGC CTT TGG AAA TGG ATG	167	60.3	50
		R: GAG GCT TGC TGG GTC GTC TT		61.3	61.6
RARG	NM_000966	F: CAA GGT CAG CAA AGC CCA TCA G	169	64.4	54.4
		R: CCG CTT GGC AAA CTC CAC GAT		66.8	57.1
RXRA	NM_002957	F: CTG TGG GTA GTC GGC TGG TGT	160	62.1	61.9
		R: GTA ACC GCT CCG CCT TTC CTG		65.8	61.9
RXRB	NM_021976	F: TTG TTG AGT GGG CGA AGA GGA T	180	63.4	50
		R: AAT GGG CTG AGT TGC GGT GC		65.4	60
RXRG	NM_006917	F: CCT CTT TCT CCC ACC GCT CAG	183	63.6	61.9
		R: CTC GCA GGC ATC CCA GTT CC		65	65

from 1 mg total RNA in 25 ml reaction buffer using MMLV reverse transcriptase, recombinant RNasin (Promega, Wallisellen, Switzerland) and random primers p(dN)6 (Roche, Rotkreuz, Switzerland) after DNase digestion with DNase I (Roche, Rotkreuz, Switzerland). The cDNA products were used for RT–PCR in a reaction mixture (25 ml) containing 12.5 ml 2 x SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and forward and reverse primer at 300 nM. The primers for RARa, RARb, RARg and RXRa, RXRb, RXRg were designed according to the NCBI reference sequences for the corresponding genes by Primer 5.0 (Table 1). 7S rRNA was used as reference target sequence (primers: 50-accaccaggttgcctaagga and 50-cacgggagttttgacctgct). We used the ABI PRISM 9700 Sequence Detection System for RT–PCR. The following parameters were for used the RT–PCR: initial denaturation (10min at 95 °C) followed by 45 amplification cycles (denaturation for 30 s at 95 °C, annealing for 30 s at 55 °C and elongation for 15 s at 72 °C). We excluded the formation of primer dimers by final melting curve analysis after RT–PCR (temperature slope 0.05 °C/s from 40 to 100 °C). All samples were measured as duplicates with a no-template control in each run. Calibration curves for each resulting plasmid were established using 10-fold serial dilutions in 50 mg/ml yeast RNA (Ambion, Huntingdon, UK). The concentration of 7S was used as a control for RNA content and for normalization of RNA content of each sample.

Eligibility, enrollment and treatment regimens

The eligibility criteria for enrollment of the total of 80 patients with gastric cancer in the present trials were as follows: diagnosis of de novo gastric cancer from January 2008 to April 2010, normal hepatic and renal function, normal blood fat and an Eastern Cooperative Oncology Group (ECOG) performance status of less than 4. Therapy for gastric cancer in this trial consisted of either the standard treatment algorithm only according to the United States NCCN Clinical Practice Guidelines in Oncology for Gastric Cancer or the standard treatment with 45 mg of oral ATRA per square meter of body-surface area for six months. No neoadjuvant chemotherapy or chemoradiotherapy was given during the study period, no major changes in the surgery and chemotherapy protocols were made during the study period, chemotherapy regimens included fluorouracil, epirubicin, cisplatin, all the resectable patients had a radical gastrectomy with level 2 extended lymphadenectomy (D2 resection).

In this treatment assignments were balanced according to nodal status, the type of surgery, and institution with the use of a biased-coin minimization algorithm (White and Freedman, 1978; Pocock and Simon, 1975) and a dynamic allocation procedure (Pocock and Simon, 1975). Data were collected and registered prospectively.

The following information was collected for each patient from the hospital archive: age, gender, date of diagnosis, presenting signs and symptoms, description of the surgical operation, radicalness of the resection performed, category of Borrmann macroscopic features at presentation, diameters of the tumor mass (tumor size), stomach subsite, number of regional metastatic lymph nodes, microscopic histological type of the tumor, depth of penetration into the gastric wall, tumor grade (cell differentiation: well, moderately, or poorly differentiated adenocarcinoma), the expressions of RARs/RXRs, ECOG performance status, main laboratory data at presentation before surgery (blood cell count, serum protein electrophoresis, and liver and kidney function tests), and type of treatment for the cohort. Informed consent was obtained from all patients. The protocol was approved by the research ethics board of the First Affiliated Hospital of Anhui Medical University according to the Declaration of Helsinki.

Statistical analysis

The Pearson's x^2-test and Mann-Whitney U test were used to analyze differences in the distribution between patient subsets of categorical and parametric and nonparametric test respectively. Multivariate analysis was performed using the Cox proportional hazards mode and included tumor stage, age at diagnosis, gender, cell differentiation, tumor size, stomach subsite, category of Borrmann and the expressions of RARs/RXRs (Cox, 1972). The global model significance was based on the x2 method and the significance of individual covariates on the Wald statistic. Overall survival was the primary end points of this study and was calculated from the date of a pathological diagnosis of de novo gastric adenocarcinomas and from the treatment of ATRA. Overall survival rates were determined using Kaplan–Meier estimator and life table method, the log-rank test was used to identify differences between the survival curves of different patients groups (Kaplan and Meier, 1958; Mantel, 1966; Peto et al., 1977). All P values reported are two-sided. For
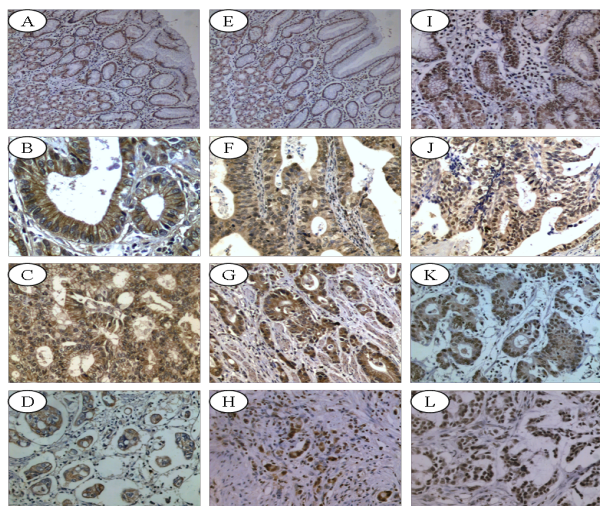
Figure 1. Representative Example Demonstrating the Immunohistochemical Staining of Retinoic Acid Receptors (RARs) a, β, and γ in Gastric Adenocarcinoma Tissue. (A-D) RARa. (A) Normal gastric epithelium (NGE): nuclear staining, strongly positive (×100). (B) Well-differentiated adenocarcinoma (WDA): cytoplasm staining, strongly positive (×400). (C) Moderate-differentiated adenocarcinoma (MDA): cytoplasm staining, strongly positive (×200). (D) Poorly differentiated adenocarcinoma (PDA): cytoplasm staining, moderately positive (×100). (E-H) RARβ. (E) NGE: nuclear staining, strongly positive (×100). (F) WDA: nuclear staining, strong positive (×200). (G) MDA: nuclear and cytoplasm staining, strongly positive (×200). (H) PDA: nuclear and cytoplasm staining, moderately positive (×100). (I-L) RARa. (I) NGE: nuclear and cytoplasm staining, strongly positive (×100). (J) WDA: nuclear staining, moderately positive (×100). (K) MDA: nuclear staining, strongly positive (×200). (L) PDA: nuclear staining, strongly positive (× 100)

Figure 2. Representative Example Demonstrating the Immunohistochemical Staining of Retinoid X Receptors (RXRs) a, β, and γ in Gastric Adenocarcinoma Tissue. (A-D) RXRa. (A) Normal gastric epithelium (NGE): cytoplasm staining, moderately positive (×100). (B) Well-differentiated adenocarcinoma (WDA): nuclear and cytoplasm staining, moderately positive (×100). (C) Moderate-differentiated adenocarcinoma (MDA): cytoplasm staining, moderately positive (× 200). (D) Poorly differentiated adenocarcinoma (PDA): nuclear staining, moderately positive (× 100). (E-H) RXRβ. (E) NGE: negative (×100). (F) WDA: negative (×200) (G) MDA: negative (×100) (H) PDA: negative (×200) (I-L) RARa. (I) NGE: nuclear and cytoplasm staining, strongly positive (×100). (J) WDA: nuclear and cytoplasm staining, moderately positive (×200). (K) MDA: cytoplasm staining, strongly positive (×100). (L) PDA: nuclear staining, strongly positive (×100)
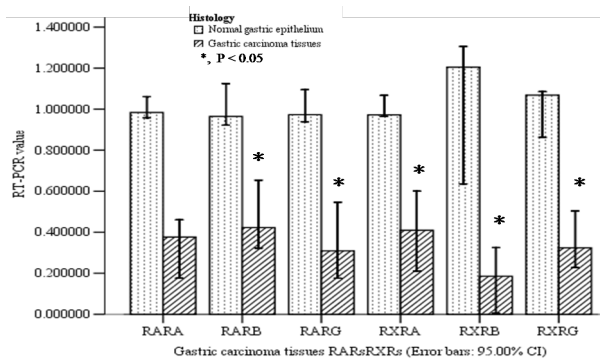

Figure 3. the mRNA Expression of RARs/RXRs in Normal Gastric Epithelium and Carcinomas

all analysis, only P < 0.05 was considered significance. All statistical analyses and graphics were performed using the statistical software SPSS version 13.

Results

Immunohistochemical study

Nuclear or cytoplasmic staining was considered positive. Nuclear or cytoplasm expression of RARa, RARβ RARγ, RXRa and RXRγ was present in carcinoma cells and normal gastric epithelium (Figure 1 and 2). Specific high immunoreactivity for RXRβ was not discernible, neither in a selection of gastric cancer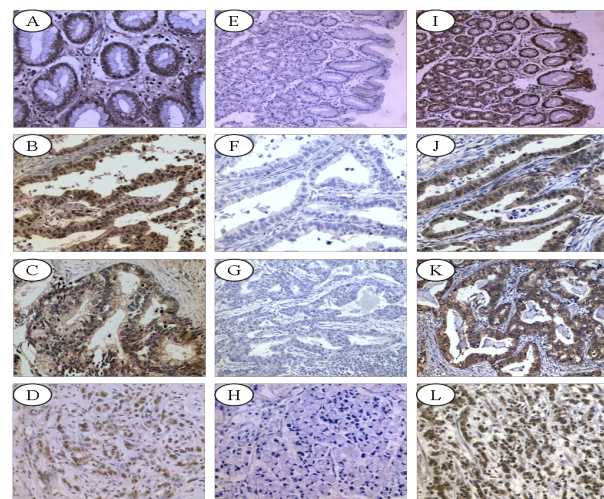s or in normal gastric tissues that were supposed to express the antigen. Then, we found in RT–PCR study that less mRNA for RXRβ was detected in frozen gastric cancer tissue. The expressions of RARa, RARβ, RARγ, RXRa, RARβ and RXRγ versus the main clinical and pathological characteristics were summarized in Table 2.

RARa, RARβ, RARγ and RXRγ had a significantly lower score in the carcinoma tissue sections than normal gastric epithelium (p = 0.022, 0.002, 0.001, < 0.001 respectively). Lower RARβ, RARγ and RXRa expressions were significantly related to advanced TNM stages (P = 0.011, 0.010, 0.013 respectively). Tumors with poor histopathologic cell differentiation had lower levels of RARa and RARβ in different histological grades of gastric carcinoma (P < 0.01). There was no significant relation between RARa, RARβ, RARγ, RXRa, RXRβ and RXRγ expressions and other clinical or pathological features. There was no difference of nuclear or cytoplasmic location among cell differentiation, Stage of TNM and Histology (data not shown). The expressions among RARa, RARβ, RARγ, RXRa and RXRγ were significantly positively correlated (P < 0.001, Table 3).

RNA expression study

The results of mRNA quantitation of the major classes of retinoid receptors are summarized in (Figure3). All the mRNA expressions of the receptors of RARa, RARβ, RARγ, RXRa, RXRβ and RXRγ were expressed in normal gastric epithelium and carcinomas, and

Table 2. Expression of Retinoid Receptor by Immunohistochemistry

	Age, year			Gender		celldifferentiation			StageofTNM			IV	Histology	
	<50	51–65	>65	male	formale	good	Intermediate	Poor	0/I	II	III	IV	normal	cancer
RARα														
0	14(41.2%)	37(50.0%)	18(46.2%)	51(45.9%)	18(50.0%)	7(22.6%)	15(41.7%)	47(58.8%)	11(39.3%)	12(33.3%)	35(53.8%)	11(61.1%)	13(25.5%)	68(46.9%)
1	9(26.5%)	18(24.3%)	9(23.1%)	25(22.5%)	11(30.6%)	9(29.0%)	12(33.3%)	15(18.8%)	4(14.3%)	11(30.6%)	17(26.2%)	4(22.2%)	14(27.5%)	36(24.5%)
2	8(23.5%)	14(18.9%)	8(20.5%)	24(21.6%)	6(16.7%)	8(25.8%)	6(16.7%)	16(20.0%)	9(32.1%)	10(27.8%)	9(13.8%)	2(11.1%)	14(27.5%)	30(20.4%)
3	3(8.8%)	5(6.8%)	4(10.3%)	11(9.9%)	1(2.8%)	7(22.6%)	3(8.3%)	2(2.5%)	4(14.3%)	3(8.3%)	4(6.2%)	1(5.6%)	10(19.6%)	12(8.2%)
P*	0.98			0.418		0.002			0.226				0.022	
RARβ														
0	9(26.5%)	23(31.1%)	10(25.6%)	30(27.0%)	12(33.3%)	6(19.4%)	8(22.2%)	28(35.0%)	3(10.7%)	5(13.9%)	23(35.4%)	11(61.1%)	3(5.9%)	42(28.6%)
1	7(20.6%)	19(25.7%)	8(20.5%)	24(21.6%)	10(27.8%)	5(16.1%)	12(33.3%)	17(21.3%)	6(21.4%)	10(27.8%)	16(24.6%)	2(11.1%)	13(25.5%)	34(23.1%)
2	7(20.6%)	15(20.3%)	17(43.6%)	31(27.9%)	8(22.2%)		14(38.9%)	18(22.5%)	10(35.7%)	13(36.1%)	13(20.0%)	3(16.7%)	13(25.5%)	39(26.5%)
3	11(32.4%)	17(23.0%)	4(10.3%)	26(23.4%)	6(16.7%)	13(41.9%)	2(5.6%)	17(21.3%)	9(32.1%)	8(22.2%)	13(20.0%)	2(11.1%)	22(43.1%)	32(21.8%)
P*	0.096			0.631		0.007			0.011				0.002	
RARγ														
0	17(50.0%)	42(56.8%)	27(69.2%)	63(56.8%)	23(65.7%)	19(61.3%)	18(50.0%)	49(61.3%)	13(46.4%)	18(50.0)	40(61.5%)	15(83.3%)	15(29.4%)	86(58.5%)
1	7(20.6%)	17(23.0%)	4(10.3%)	20(18.0%)	8(22.9%)	3(9.7%)	10(27.8%)	15(18.8%)	4(14.3%)	8(22.2%)	16(24.6%)	0	10(19.6%)	28(19.0%)
2	8(23.5%)	9(12.2%)	5(12.8%)	17(15.3%)	4(11.4%)	1(3.2%)	7(19.4%)	14(17.5%)	5(17.9%)	8(22.2%)	8(12.3%)	1(5.6%)	16(31.4%)	22(15.0%)
3	2(5.9%)	6(8.1%)	3(7.7%)	11(9.9%)	0	8(25.8%)	1(2.8%)	2(2.5%)	6(21.4%)	2(5.6%)	1(1.5%)	2(11.1%)	10(19.6%)	11(7.5%)
P*	0.448			0.252		0			0.01				0.001	
RXRα														
0	13(38.2%)	33(44.6%)	17(42.5%)	46(41.4%)	16(44.4%)	9(29.0%)	15(41.7%)	38(47.5%)	8(28.6%)	11(30.6%)	31(47.7%)	12(66.7%)	12(23.5%)	62(42.2%)
1	8(23.5%)	18(24.3%)	11(27.5%)	22(19.8%)	15(41.7%)	6(19.4%)	12(33.3%)	19(23.8%)	4(14.3%)	10(27.8%)	21(32.3%)	2(11.1%)	15(29.4%)	37(25.2%)
2	7(20.6%)	13(17.6%)	10(25.0%)	27(24.3%)	3(8.3%)	8(25.8%)	8(22.2%)	14(17.5%)	9(32.1%)	11(30.6%)	8(12.3%)	2(11.1%)	13(25.5%)	30(20.4%)
3	6(17.6%)	10(13.5%)	2(5.0%)	16(14.4%)	2(5.6%)	2(5.6%)	1(2.8%)	9(11.3%)	7(25.0%)	4(11.1%)	5(7.7%)	2(11.1%)	11(21.6%)	18(12.2%)
P*	0.707			0.056		0.076			0.013				0.09	
RXRβ														
0	29(85.3%)	63(85.1%)	32(82.1%)	94(84.7%)	30(83.3%)	23(74.2%)	28(77.8%)	73(91.3%)	21(75.0%)	29(80.6%)	57(87.7%)	17(94.4%)	43(84.3%)	120(84.4%)
1	2(5.9%)	5(6.8%)	4(10.3%)	7(6.3%)	4(11.1%)	5(16.1%)	4(11.1%)	2(2.6%)	2(7.1%)	3(8.3%)	5(7.7%)	1(5.6%)	7(13.7%)	11(7.5%)
2	3(8.8%)	6(8.1%)	3(7.7%)	10(9.0%)	2(5.6%)	3(9.7%)	4(11.1%)	5(6.3%)	5(17.9%)	4(11.1%)	3(4.6%)	0	1(2.0%)	12(8.2%)
3	0	0	0	0	0	0	0	0	0	0	0	0	0	0
P*	0.959			0.537		0.084			0.333				0.146	
RXRγ														
0	13(38.2%)	34(48.6%)	20(51.3%)	51(45.9%)	18(50.0%)	14(45.2%)	15(41.7%)	40(50.0%)	12(42.9%)	13(36.1%)	33(50.8%)	11(61.1%)	4(7.8%)	69(46.9%)
1	8(23.5%)	15(21.4%)	8(20.5%)	25(22.5%)	8(22.2%)	9(29.0%)	10(27.8%)	14(17.5%)	5(17.9%)	9(25.0%)	15(23.1%)	4(22.2%)	14(27.5%)	33(22.4%)
2	8(23.5%)	9(12.9%)	7(17.9%)	18(16.2%)	6(16.7%)	3(9.7%)	7(19.4%)	14(17.5%)	7(25.0%)	9(25.0%)	7(10.8%)	1(5.6%)	13(25.5%)	24(16.3%)
3	5(14.7%)	12(17.1%)	4(10.3%)	17(15.3%)	4(11.1%)	5(16.1%)	4(11.1%)	12(15.0%)	4(14.3%)	5(13.9%)	10(15.4%)	2(11.1%)	20(39.2%)	21(14.3%)
P*	0.755			0.933		0.704			0.535				0	

*Pearson's x^2-test; RAR, retinoicacidreceptor; RXR, retinoid X recept; 0, negative; 1, lightpositive; 2, moderatepositive; 3, strongpositive

Table 3. The Correlated Correlation among RARs and RXRs

	RARα	RARβ	RARγ	RXRα	RXRβ	RXRγ
RARα Correlation coefficient	1.000	0.506	0.533	0.540	0.394	0.358
P	0.000	0.000	0.000	0.000	0.000	0.000
RARβ Correlation coefficient		1.000	0.458	0.578	0.248	0.591
P			0.000	0.000	0.002	0.000
RARγ Correlation coefficient			1.000	0.471	0.471	0.588
P				0.000	0.000	0.000
RXRα Correlation coefficient				1.000	0.333	0.558
P					0.000	0.000
RXRβ Correlation coefficient					1.000	0.321
P						0.000
RXRγ Correlation coefficient						1.000

Spearman rank correlation; RAR, retinoic acid receptor; RXR, retinoid X recept

the mRNA expressions of retinoid receptors were significantly lower in gastric carcinoma tissues than in normal gastric epithelium (Mann-Whitney U test, Z=2.324, P=0.020). They informed us the genetic transcriptions levels of RARs/RXRs had changed lowly in the gastric cancer. This observation may imply that the abnormally lower expressions of retinoid receptors on the genetic transcriptions levels have some relation to the development and/or progression of gastric cancers.

Prognostic analyses

147 patient ages ranged from 23 to 81 years (median age 58 years). We obtained follow-up information through telephone interviews with the patients or their close relative or from communitywide physician, or the National Organization for Civil Registration. Patients were followed for a median period of 37 months (range, 1 to 128 months) from diagnosis. The 80 patients enrolled the treatment trials whose disease characteristics were examined to establish their relationship to overall survival (OS) after a median follow-up of 21 months and a maximum of 44 months from the date of diagnosis until their death or end of follow-up.

Many studies have reported on the prognostic importance of several clinicopathological factors, such as age, gender, macroscopic type (Borrmann I, II, III, and IV), tumor size, cell differentiation and TNM classification (Gobbi et al., 2011; Jiang et al., 2011; Khedmat et al., 2011), therefore, the multivariate analysis which included the expressions of RARa, RARβ, RARγ, RXRa, RXRβ and RXRγ and the aforesaid clinical variables was performed using the Cox proportional hazards mode. The score of the expressions of RARs/RXRs from 1 to 3 calculated RARs/RXRs positive, while the score of the expression of RARs/RXRs zero calculated RARs/RXRs negative. The RARa and Stage of TNM in addition to
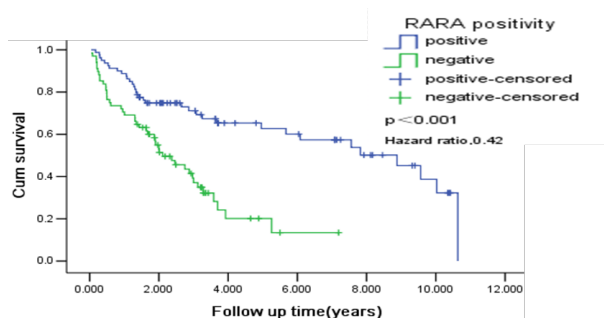
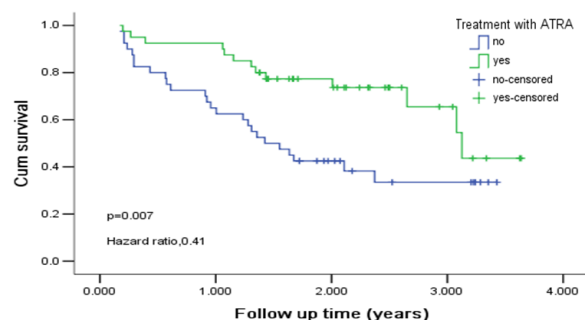**Figure 4. Overall Survivals by RARa Immunohistochemistry**

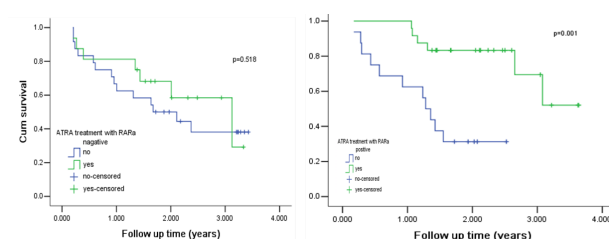**Figure 5. Overall Survivals by ATRA Treatment in 80 Patients with Gastric Cancer**

Figure 6. The Overall Survival of ATRA Treatment Between RARa Positive and RARa Negative in Gastric Cancer

RARβ, RARγ, RXRa, RXRβ, RXRγ, cell differentiation, tumor site, Borrmann style, tumor size, age and gender were independent and key predictors of survival by the method of Forward: LR (p<0.001), then overall survival (OS) was calculated using Kaplan–Meier estimator, the positive expression of RARa indicated a lower risk of death from gastric cancer (p<0.001, HR = 0.42, 95.0% CI for HR 0.24-0.73). Patients with RARa positive cancers had median survival time of 8.88 years (95.0% CI 5.85-11.91), whereas with RARa negative cancers the median survival time was 2.11 years (95.0% CI 1.31-2.91) (Figure 4)

Last, we treated the 80 patients in de novo gastric cancer with or without ATRA; overall survival was the primary end points of this study. Patients with ATRA treatment had median survival time of 3.12 years (95.0% CI 2.52-3.73), whereas without ATRA treatment the median survival was 1.42 years (95.0% CI 0.92-1.93) (p=0.007, HR = 0.41, 95.0% CI 0.21-0.80), the 3 years overall survival with ATRA treatment was 64%, however, without ATRA treatment was 34% (Figure 5). The overall survival of ATRA treatment had significant difference between RARa positive and RARa negative in gastric cancer (Figure 6).

Discussion

Despite substantial advancement of the surgical and medical therapy, gastric cancer continues to be a highly lethal disease. Even with state-of-the-art surgery and modern chemotherapy, the probability of five-year survival in the USA, Europe, and China generally is only 20–25% (Parkin, 2001).

The physiological functions of retinoids include the control of proliferation, apoptosis and differentiation in normal cells during growth and development by various mechanisms (Altucci and Gronemeyer, 2001; Huang et al., 2002; Mehta, 2003; Yoshida et al., 2003), interference with cell cycle control (Chao et al., 1997) and cross-talk with other signaling molecules such as AP1 (Wu et al., 2002) and mitogen-activated kinase signal pathways (Huang et al., 2002; Crowe et al., 2003). The expression of RARβ is induced by liganded RARa and is lost as an early event in the carcinogenesis of various tumors (Soprano et al., 2004). Most of the results from experimental animal studies indicate that retinoids are effective in preventing or suppressing cancers. Most biological effects of retinoids are mediated through retinoid receptors; previous work has established that retinoid receptors are expressed in a variety of gastric cancer cell lines (Shyu et al., 1995; Liu et al., 1998; Jiang et al., 1999; Liu et al., 2001; Lin et al., 2004; Ye et al., 2004; Karam et al., 2005). However, the expression of retinoid receptors has hardly been investigated in clinical gastric cancer tissues. In the present study, the population in this study involved all patients treated at our research institution between 2001 and 2010 for whom tissues blocks were available, we observed that the retinoid receptors RARa, RARβ, RARγ, RXRa and RXRγ were present in a majority of gastric cancer tissues, but there were significantly lower expression levels of RARa, RARβ, RARγ and RXRγ than in normal gastric epithelium, especially lower expressions of RARβ, RARγ and RXRa in advanced-stage tumors and those of RARa and RARβ with poor differentiation. High expression of RXRβ did not exist not only in gastric cancers but also in normal gastric mucosal tissues. These results could be concluded from proteinaceous levels by immunohistochemistry and genetic transcriptive levels by RT-PCR in the present study. According to previous study (Hayashi et al., 2001; Kim et al., 2004; Wu et al., 2004; Koike et al., 2005; Shutoh et al., 2005; Ben Ayed-Guerfali et al., 2011), the lower expressions of retinoid receptors RARa, RARβ, RARγ, RXRa and RXRγ were synthetically caused by DNA methylation and genetic acetylation of genetic transcriptive levels and the ubiquitin/proteasome pathway and the sumoylation pathway of proteinaceous levels. The results also revealed the expressions of RARa and RARβ maybe act as the markers of the cell differentiation in different histological types of gastric carcinoma, the poorer on histopathologic grade, the lower on expressions of RARa and RARβ.

We further detected that the low expression of RARa independently predicts an unfavorable prognosis in addition to established prognostic factors such as AJCC stage in this study. It is thus not surprising that the high

expression of RARa was a positive prognostic factor of survival. Converse observations were made in oral squamous cell carcinoma, where RARa was an independent indicator of a poor prognosis (Chakravarti et al., 2003). RARa was correlated with unfavorable prognostic factors such as high grade and high proliferation rate in prostate (Gyftopoulos et al., 2000) and breast (van der Leede et al., 1996) cancer.

All-trans retinoic acid was one of the first examples of 'targeted' therapy: it induces remissions in a majority of patients with promyelocytic leukemia by differentiation therapy (Sachs, 1978a; 1978b; Petrie et al., 2009). Moreover, retinoids prevent the development of solid tumors and are also effective in the therapy of certain solid tumors (Reynolds, 2000; Lippman et al., 2001; Abu et al., 2005; Coelho et al., 2005; Pasquali et al., 2006; Zanardi et al., 2006; Khuri et al., 1997; Riecken and Rosewicz, 1999). This interesting biological activity profile of retinoids has prompted investigators to study them in the chemoprevention of epithelial cancers and in the treatment of advanced cancers (Lippman et al., 1994; Sankaranarayanan and Mathew, 1996). Although the therapeutic response of retinoids against advanced cancers is disappointing, retinoids interact in vitro with conventional cytotoxic drugs such as cisplatin and the taxanes paclitaxel and docetaxel to lower the threshold of apoptosis (Jozan and Lafon, 1996; Aebi et al., 1997; Wang et al., 2000; Nehme et al., 2001; Jozan et al., 2002; Wang and Wieder, 2004; Garattini et al., 2007). This study had investigated that ATRA could significantly prolong OS of the patients with gastric cancer combined conventional chemotherapy with a substantially better benefit to toxicity ratio and the expression of RARa was correlated with the responsiveness to ATRA. It was conceivable that a more selective use of retinoids exclusively in tumors that expressed RARa may be achieved more in combination with conventional chemotherapy on the patients with gastric cancer. Retinoids could induce mild, dose-related anemia and dose-related increases in serum alkaline phosphatase, cholesterol, triglycerides, and calcium levels, and increased platelet counts (Horn et al., 2007). The clinical side-effects of retinoids in present study do not overlap with the typical toxicity of cytotoxic chemotherapeutic agents. Thus, clinical evidence made retinoids attractive investigational agents for the combination with conventional cytotoxic chemotherapy in gastric cancer.

In the present study, the expression among RARa, RARβ, RARγ, RXRa and RXRγ was significantly positively correlated. The presence of RXRs, although not of independent prognostic value in this study, may also be important: RXRs are obligatory dimerization partners for other members of the steroid receptor superfamily such as other retinoid receptor subtypes, the thyroid hormone receptor, the peroxisome proliferator-activated receptor gamma (PPARg), the orphan receptor and the vitamin D receptor.

Although the present investigation is limited by the small number of samples analyzed, it reveals that certain retinoid receptors are present in human gastric cancer. RARa may be associated with a positive prognosis

independently of established prognosticators such as AJCC stage, and the patients with gastric cancer treated with ATRA combined with conventional cytotoxic chemotherapy could prolonged the OS significantly. This finding will have to be verified in a larger independent set of tumor samples. The present study provides the molecular basis for clinical trials to evaluate the efficacy of retinoids in gastric cancer; this prospect is promising in view of preclinical data that show a synergic interaction with fluorouracil, epirubicin, cisplatin drugs. Retinoids are potential candidates for new treatment strategies for gastric cancer.

Acknowledgements

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