Original Paper

The OPN Gene Polymorphism Confers the Susceptibility and Response to Ara-C Based Chemotherapy in Chinese AML Patients

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Key Words

Osteopontin • Acute myeloid leukemia • Chemotherapy • Susceptibility • Polymorphism

Abstract

Aim: To investigate the possible association between the Osteopontin (OPN) gene polymorphisms and the susceptibility and chemotherapy response of acute myeloid leukemia patients. **Methods:** A total of 381 patients with de nova AML and 430 healthy controls were enrolled. All patients received Ara-C-based standard induction chemotherapy regimens, and the treatment response was evaluated. Several polymorphisms in the human OPN encoding gene have been identified. The OPN) gene polymorphisms at 3 loci, namely, -156 GG>G, -443 C>T and -66T>G were determined. **Results:** We identified that the -443C>T polymorphism was the only one which is closely related to AML. Compared with the -443TT carriers, our data showed that the -443CC genotype carriers were significantly related to a higher risk for AML. The -443CC genotype was also more prevalent in poor response groups than in good response group. The -443CC carriers are more likely to have poor response to AML treatment. Cellular assay indicated that the leukemic cell lines receiving the OPN -443C transfection have a significantly lower apoptosis rate to Ara-C treatment compared to cell lines transfected with -443T. **Conclusion:** The findings of this study suggest OPN-443C>T gene polymorphism may be sued as a molecular for susceptibility and chemotherapy response of AML.

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Introduction

Acute myeloid leukemia is a heterogeneous clonal disorder characterized by the uncontrolled proliferation of neoplastic hematopoietic precursor cells that lose the ability to differentiate into mature cells [1, 2]. The etiology of the disease is largely unknown. Currently, it is accepted that the environmental factors, including ionizing radiation, benzene

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exposure and cytotoxic chemotherapy play important role in the AML incidence [3, 4]. In addition, the genetic factors are involved in the pathogenesis of AML [5, 6]. Besides the fusion genes derived from chromosomal translocations, numerous gene mutations have also been reported in different types of AML. The genetic polymorphisms of several candidate genes have been reported to correlate to the susceptibility to AML in different ethnic populations [7, 8]. Characterizing molecular mutations in AML is important for the management of patients individually.

Osteopontin (OPN) is a sibling glycoprotein that was first identified in osteoblasts [9]. As a multi-functional cytokine, OPN is involved in cell survival, migration and adhesion which is associated with tumorigenesis, progression and metastasis. OPN is also implicated in tumorigenesis and has been proposed as a cancer marker including nasopharyngeal carcinoma, lung cancer, colon cancer and prostate cancer [10, 11].

The association between OPN and leukemia has also been reported. Adhesion to OPN in the bone marrow niche regulates lymphoblastic leukemia cell dormancy [12]. OPN increases the expression of $\beta 1$, 4-galactosyltransferase-I and promotes adhesion in human RL95-2 cells [13]. OPN also increases CD44 expression and prompts cell adhesion in RAW 264.7 murine leukemia cells [14]. Clinically, OPN is used as a prognostic factor for survival of AML patient [15].

Several polymorphisms in the human OPN encoding gene have been identified. The -66T>G (rs28357094), -156G>/GG (rs17524488), and -443C>T (rs11730582), polymorphisms among the most studied loci. The OPN gene polymorphism has been documented to be related to the susceptibility to cervical and thyroid cancers and their clinical features as well as prognosis [16, 17]. To date, whether the OPN gene polymorphism affects the susceptibility to AML remains unknown. Base on the increased expression of OPN in the AML, we postulate there might be a positive association between the OPN gene polymorphism and AML incidence. Also we investigate if the OPN gene polymorphism affects the chemotherapy response in these patients.

Materials and Methods

A total of 381 patients with de nova AML without previous treatment were consecutively enrolled in this study. The diagnosis of AML was performed according to clinical, morphological, cytochemical, and immunophenotypic examination. Among these patients with AML, 4 patients were diagnosed with minimally differentiated AML (M0); 38 patients for AML without maturation (M1); 130 AML with maturation (M2); 78 patients acute myelomonocytic leukemia (M4); 115 patients, acute monocytic leukemia (M5); 12 patients, erythroleukemia (M6); and 5 patients, acute megakaryoblastic leukemia (M7). The characteristics of the patients are shown in Table 1. The acute promyelocytic leukemia (M3) was excluded because of different treatment regimens. A total of 430 healthy subjects were enrolled as controls as well. Karyotype analysis showed that 172 had normal karyotype and 209 cases had karyotypic abnormalities: 46 had +8, 39 had -7/del, 33 had +10, 12 had abn(11), 4 had abn(17), 3 had abn (19), 41 had t(8,21) and 31 had high complex karyotype. The controls and AML patients were matched for age and gender. All participants were ethnically of Chinese Han. This study was approved by the Institute's Review Board and conducted according to the Helsinki declaration. The written consent was obtained from all patients and healthy controls and

Evaluation of chemotherapy regimens and therapeutic response

All patients received Ara-C-based standard induction chemotherapy regimens, and the treatment effects were evaluated after the second cycles of chemotherapy regimen. Patients receiving allogeneic stem cell transplantation were excluded from this study. A total of 115 patients received intravenous daunorubicin (DNR) 45 mg $\times$ m-2 $\times$ d-1 for 1-3 days and Ara-C 100 mg $\times$ m-2 $\times$ d-1 for 1-7 days (DA induction chemotherapy regimen); 167 patients received homoharringtonine (HHT) 3-4 mg $\times$ m-2 $\times$ d-1 for 5-7 days and Ara-C 100 mg $\times$ m-2 $\times$ d-1 for 1-7 days (HA induction chemotherapy regimen); and 99 patients received mitoxantrone 4 mg $\times$ m-2 $\times$ d-1 for 1-5 days and Ara-C 100 mg $\times$ m-2 $\times$ d-1 for 1-7 days (MA induction chemotherapy regimen). Complete response (CR) was defined as follows: absolute values of granular leukocytes and platelets in

peripheral blood are not more than $1.5 \times 10^9/L$ and $100 \times 10^9/L$ respectively; blast cell count in the bone marrow was less than 5% for at least 4 weeks and symptoms and signs of leukemia disappear; and granulopoiesis and megakaryocytopoiesis after chemotherapy with normalized peripheral blood counts persisting for at least 4 weeks, without intervening chemotherapy. Patients with other treatment response, including Partial remission, Non-remission and Early death were assigned as non-CR group [18].

OPN gene polymorphisms

DNA was extracted from peripheral whole blood using a Qiagen DNA Isolation Kit (Qiagen, Valencia, CA, USA). Three single nucleotide polymorphisms in the promoter region of OPN gene, including -66T>G (rs28357094), -156G>/GG (rs17524488), and -443C>T (rs11730582), were determined using the TaqMan 5' allelic discrimination assay. It was performed using a commercially available kit Assays-on-Demand™ SNP genotyping products (Applied Biosystems, Foster City, CA). SNP amplification assays were used according to the manufacturer's instructions. In short, 10 ng of sample DNA in 25 μL of reaction solution containing 12.5 μL of the 2 $\times$ TaqMan® Universal PCR Mix (Applied Biosystems), and 1.25 μL of pre-developed assay reagent from the SNP genotyping product containing two primers and two MCB-Taqman probes. Reaction condition was consisted of pre incubation at 50 °C for 2 min, at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Amplifications were performed in an ABI Prism® 7500 Sequence Detection System (Applied Biosystems).

Construction of luciferase reporter plasmids

A 644/643 bp fragment from -645 to -2 bp of the OPN promoter was prepared by PCR amplification from genomic DNA of homozygous individuals for the four most common haplotypes (forward primer: 5'-ggggtaccTGAAGCAGCCCTCTCAAGCA-3'; reverse primer: 5'-gaagatctACAACCAAGCCCTCCAGAAT-3'). DNA fragments containing different alleles of the human SPP1 gene from -645 to -2 (-155_156G/-443T, -155_156G/-443C, -155_156GG/-443T and -155_156GG/-443C) were cloned into the pGL3 Basic Vector (Promega, Madison, WI, USA) between KpnI and BglII restriction sites, which were designated as A, B, C and D plasmids, respectively [19].

Cell culture, transfection and luciferase assay

Four leukemic cell lines, namely, KG1a, Kasumi-1, U937 and Thp-1 were cultured in a humidified incubator with 5% CO₂ at 37 °C. One day before transfection, the cells were cultured in a 96-well plate in 100 μL culture medium without antibiotics to 90–95% confluence at the time of transfection. The cells were transfected using Lipofectamine 2000 Reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). Briefly, 0.2 μg of the pGL3 vector containing an OPN promoter fragment was used for each well. The transfection effect was >90%.

Western Blotting Analysis

Collected the transfected cells for western blot assay to detect OPN expression levels in these cells. Cells were lysed by RIPA Lysis Buffer. The protein concentration in the supernatant was determined with a BCA protein assay kit. The isolated protein (15 μg) was separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride nylon membranes. The blots were probed with mouse anti-OPN, and anti-GAPDH antibody (1: 1000 dilution; Santa Cruz Biotechnology, Santa Cruz, USA). Then with horseradish peroxidase-conjugated secondary antibodies (1: 1000 dilution), and visualized using an ECL detection kit (Amersham Biosciences, Piscataway, NJ, USA).

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Assay

The cell lines receiving different allele constructs transfections were treated with Ara-C at concentrations of 1.5 μM for 24h. The apoptotic cells were detected by in situ DeadEnd™ Colorimetric Apoptosis Detection System (Promega, Madison, WI) according to the manufacturer's instructions. Briefly, sample slides were washed in PBS and then fixed in 4 % paraformaldehyde solution. Sections were incubated with terminal deoxynucleotidyl transferase enzyme in a humidified chamber at 37 °C for 60 min. The reaction was terminated by transferring the slides to 2 $\times$ sodium citrate saline solution. The sections were counter stained with DAPI. For quantitative analyses, 5 fields per section were selected. Apoptosis was indexed by counting TUNEL positive cells per 100 nuclei per section.

Table 1. The characteristics from AML patients and controls

Characteristic	Cases N=381	Controls N =430	P value
Sex			
Male	197	224	0.484
Female	184	206	
Age (years)	54.5±3.6	54.6±4.1	0.763
Smoke status			<0.001
never	56	146	
ever smoker	146	143	
current	179	141	

Statistical Analysis

Deviations from Hardy-Weinberg equilibrium (HWE) for genotypes were assessed by χ^2 -test. Demographic and clinical information was compared to genotype, using χ^2 -test or Fisher's exact test. The significant difference of genotypes in different responders was calculated using χ^2 test. We estimated the risk for AML as odds ratio (ORs) and 95% confidence intervals (CIs). The OR and 95% CI were calculated by Logistic regression analysis with adjustment of clinical variables. All statistical tests for this analysis were performed using SPSS 17.0 software (SPSS Inc., USA). P values lower than 0.05 were considered statistically significant.

Results

The characteristics from patients are presented in Table 1. There was no significant difference in age and sex between AML cases and controls (all $P > 0.05$). The AML patients have higher percentage of smokers than controls ($P < 0.001$).

Table 2 describes the genotype and allele distributions of OPN gene polymorphisms in the AML and control groups. The genotype frequencies for all polymorphisms did not differ significantly from those expected under Hardy-Weinberg equilibrium (all $P > 0.05$). For the -443 C>T polymorphism, AML patients had a higher prevalence of CC genotype than the controls (30.71% vs. 20.93%, global $P < 0.001$). We performed the univariate and multivariate logistic regression analysis with the adjustment of age, sex, smoking status, white blood cells, hemoglobin and platelet numbers. Compared with the -443TT carriers, our data showed that the -443CC genotype carriers were significantly related to a higher risk for AML (adjusted OR=1.98, 95%CI: 1.45-2.89 adjusted $P=0.002$). The -443C allele carriage is associated with higher risk for AML (adjusted OR=1.44, 95%CI: 1.05-1.52 adjusted $P=0.015$). In contrast, the genotype distribution of -156G>GG and -66G>T were not significantly different between the AML and control groups (both $P > 0.05$). The allelic frequencies in -66T/-66G and -156G/-156GG were not similar between AML patients and healthy controls. The logistic regression analysis did reveal the association of genotype and allele distributions of -156G>GG and -66G>T with the incidence of AML (Table 2).

The mean serum OPN levels were compared according to the OPN genotypes. We found that the -443CC carriers had a significantly higher serum OPN than CT and TT carriers (194 ± 12 vs. 129 ± 21 and 114 ± 13 , ng/ml, $P < 0.001$). The gene polymorphisms at other -156G>GG and -66G>T did not affect the thrombin-cleaved OPN levels.

We next stratified all patients and controls by age (<60 years old and ≥ 60 years old). We found that the -443C allele carriage is still associated with higher risk for AML (adjusted OR=1.95, 95%CI: 1.12-3.40, adjusted $P=0.018$) in <60 group and ≥ 60 group (Table 3).

Table 4 showed the frequencies of genotypes in different treatment response in AML patients, and the association of OPN genotypes with the treatment response status. The -443CC genotype was more prevalent in non-CR groups than in CR group. After adjusting with clinical variables including age, sex, smoking status, white blood cells, hemoglobin and platelet numbers and distribution of chemotherapy agents, the multiple regression analyses revealed that the -443CC carriers are more likely to have poor response to AML treatment (adjusted OR=2.09, $p=0.023$, Table 3). For other loci of OPN gene, the genotypes were not

Table 2. The genotype and allele distributions of OPN gene polymorphisms in AML and control groups

		AML N=381	%	Control N=430	%	adjusted OR	95%CI	adjusted P
-66T>G	TT	89	23.36%	99	23.02%	1.00		
	TG	199	52.23%	210	48.84%	1.05	0.75 1.49	0.765
	GG	93	24.41%	121	28.14%	0.85	0.58 1.27	0.435
	T	377	49.48%	543	51.03%	1.00		
	G	385	50.52%	521	48.97%	1.06	0.88 1.28	0.511
-443C>T	TT	81	21.26%	117	27.21%	1.00		
	CT	183	48.03%	223	51.86%	1.19	0.84 1.67	0.333
	CC	117	30.71%	90	20.93%	1.98	1.45 2.89	0.002
	T	345	45.28%	543	51.03%	1.00		
	C	417	54.72%	521	48.97%	1.44	1.05 1.52	0.015
-156G>GG	GG	84	22.05%	114	26.51%	1.00		
	GGG	198	51.97%	226	52.56%	1.19	0.85 1.67	0.319
	GGG	99	25.98%	90	20.93%	1.49	1.00 2.23	0.050
	G	366	48.03%	543	51.03%	1.00		
	GG	396	51.97%	521	48.97%	1.13	0.94 1.36	0.206

Table 3. The genotype and allele distributions of OPN gene polymorphisms in AML and control groups stratified by age

Age	Genotype	AML		Control		adjusted OR	95%CI	adjusted P
<60	-443C>T	TT	40	21.39%	58	28.29%	1.00	
		CT	91	48.66%	102	49.76%	1.29	0.79 2.12
		CC	56	29.95%	45	21.95%	1.80	1.03 3.17
		T	171	45.72%	218	53.17%	1.00	
		C	203	54.28%	192	46.83%	1.35	1.02 1.79
≥60	-443C>T	TT	41	21.13%	59	26.22%	1.00	
		CT	92	47.42%	121	53.78%	1.09	0.68 1.77
		CC	61	31.44%	45	20.00%	1.95	1.12 3.40
		T	174	44.85%	239	53.11%	1.00	
		C	214	55.15%	211	46.89%	1.39	1.06 1.83

Table 4. The frequencies of genotypes of -443C>T genotype in treatment response to patients with AML

		Non-CR	%	CR	%	adjusted OR	95%CI	adjusted P
-443C>T	TT	43	23.24%	54	27.55%	1.00		
	CT	82	44.32%	104	53.06%	0.99	0.60 1.62	0.969
	CC	60	32.43%	38	19.39%	1.98	1.12 3.51	0.018
	T	168	45.41%	212	54.08%	1.00		
	C	202	54.59%	180	45.92%	1.42	1.06 1.88	0.017

substantially different between good response and poor response groups (data not shown).

By using the Dual Luciferase Reporter Assay System we compared the promoter activity of leukemia cell lines transfected with different OPN gene alleles. We found a significant difference between cells transfected with the -443C and -443 T alleles. As shown in Figure 1a, significantly higher luciferase activities were observed with the pGL3-C construct compared to the pGL3-T construct (all $P < 0.001$ in 4 cell lines). As expected, Figure 1b shows that the -443C construct transfection induced significantly higher OPN protein expressions in all tested leukemic cell lines.

The cell lines receiving different allele constructs transfections were treated with Ara-C, followed by TUNEL assay to evaluate the cellular apoptosis levels. Cellular assay indicated that only the -443C and -443T transfection significantly affect the proliferation and apoptosis rates of leukemic cell lines induced by Ara-C. The leukemic cell lines receiving the OPN -443C transfection have a lower apoptosis rate to Ara-C treatment compared to cell lines transfected with -443T. Figure 2 shows the quantitative analyses of the apoptosis rates in the studied cell lines.

Fig. 1. a) The OPN gene promoter activities and OPN protein expression in cultured leukemia cell lines transfected with pGL3-T and pGL3-C construct. The -443C construct transfection induced significantly higher promoter activities in all tested leukemic cell lines ($\dagger$, $P < 0.001$, vs. -443T). b) The -443C construct transfection induced significantly higher OPN protein expressions in all tested leukemic cell lines.

Fig. 2. The quantitative analyses of the apoptosis rates in cell lines transfected with pGL3-T and pGL3-C construct. The -443C construct transfection increased the resistance of leukemic cell lines to Ara-C treatment, indicated by significantly reduced apoptosis rates ($\dagger$, $P < 0.001$, vs. -443T).

Discussion

In the present study, we reported the association between OPN gene polymorphism and AML in Chinese patients. We identified that the -443C>T polymorphism was the only one which is closely related to AML. Compared with the -443TT carriers, our data showed that the -443CC genotype carriers were significantly related to a higher risk for AML. The -443CC genotype was also more prevalent in poor response groups than in good response group. The -443CC carriers are more likely to have poor response to AML treatment. Cellular assay indicated that the leukemic cell lines receiving the OPN -443C transfection have a significantly lower apoptosis rate to Ara-C treatment compared to cell lines transfected with -443T. To the best of our knowledge, this is the first study regarding the close association between OPN-443C>T gene polymorphism and the susceptibility and chemotherapy response of AML.

Osteopontin (OPN) is a secreted glycoprotein of the SIBLING family that is involved in physiologic and pathophysiologic processes. The association between OPN and solid cancers has been reported in hepatocellular carcinoma, pancreatic Cancer and lung cancer [20-22]. Increased serum OPN concentrations occur in chronic myeloid leukemia, multiple myeloma, and acute myeloid leukemia [23]. Higher OPN were associated with significant shorter survival times in AML patients in a 1-year survival analysis [24].

The OPN gene polymorphism has been documented to be related to risk, clinical feature and prognosis of a variety of cancers. For example, the cervical cancer patients had

markedly higher percentage of -156 GG carriage and significantly lower TT and TC of -443 genotypes than controls [17]. Variation at -443 in the OPN promoter increases the potential for gastric cancer metastasis and subsequent death in the Chinese population [25]. In that study, the authors measured the promoter activity of the -443 T>C polymorphism using a dual luciferase reporter assay. Significantly higher luciferase activities were observed in the pGL3-C construct compared to the pGL3-T construct [25]. In gliomas patients, those patients with -443CC genotype had a poorer prognosis than those with -443TT and -443CT genotypes [26]. OPN -443C>T gene polymorphism may be used as a molecular marker to predict the treatment response to chemotherapy in advanced lung cancer patients [27]. OPN plays a crucial role in hepatocellular carcinoma (HCC) metastasis. The genetic variation at locus -443 of the OPN promoter plays important roles in the regulation of OPN expression and cancer progression of HCCs [28]. In this study, we found that the -443C>T OPN-443C>T gene polymorphism and the susceptibility and chemotherapy response of AML. This is consistent with most of the published studies. A recent study shows that AML patients expressing high levels of OPN within the bone marrow experienced shortened overall survival [15]. The -443CC carriers had significantly higher OPN than CT and TT carriers. We thus postulate the OPN gene polymorphism affect the risk and prognosis or AML patients via regulation of OPN protein levels.

The association between the OPN-443C>T gene polymorphism and the chemotherapy response is another novelty of this study. To date, only a few studies reported OPN gene polymorphism as a marker predicting the efficacy of interferon-based therapies in patients with chronic hepatitis C [29], but no study reported the OPN gene polymorphism and the chemotherapy response in cancer. In this study, we found that -443CC, not only increases the AML risk, but also make its carriers more resistant to chemotherapy. This observation is supported by our in vitro study. We found that the leukemic cell lines receiving the OPN -443C transfection have a significantly lower apoptosis rate to Ara-C treatment compared to cell lines transfected with -443T. We postulate the OPN-443C>T gene polymorphism may influence the transcript activity and affect the OPN expression level, thus change the cellular response to Ara-C induced apoptosis.

In this study, we observe a higher smoking rate among AML patients compared with healthy controls. Previous cohort and case-control studies have suggested that cigarette smoking may be involved in the etiology of leukemia in many populations [30-32]. Cigarette smoke has also been linked to adult myeloid leukemia. Exposure to paternal preconception smoking alone or in combination with postnatal passive smoking increases the risk of childhood leukemia [33]. To investigate the effect of gene-environment interactions to incidence of myeloid leukemia, Vineis et al. selected several candidate polymorphisms in genes coding for enzymes involved in biotransformation of environmental toxicants and found that in people exposed to high levels of environmental tobacco smoke, the studied genetic genotype (GSTM1, GSTT1, SULT 1A1) was associated with an increased risk of leukemia [34]. In this study, we involved the smoking status in the univariate and multivariate logistic regression analysis to see if the higher AML prevalence rate with certain polymorphism is associated with smoking incidence. Our data showed that the -443CC genotype carriers were significantly related to a higher risk for AML (adjusted OR=1.98, 95%CI: 1.45-2.89 adjusted P=0.002). However, smoking itself is not an independent risk factor for incidence of AML in our study.

Several limitations in this study need to be addressed. This study was a single-center cohort investigation and the sample size is relatively small. In addition, all the participants of this study were Chinese. Thus, the association between OPN gene polymorphism and AML needs to be further tested in different ethnic populations with larger sample size. Secondly, a recent study shows that AML patients expressing high levels of OPN within the bone marrow experienced shortened overall survival [15]. We did not provide the prognostic data of OPN gene polymorphism on AML patients.

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