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RESEARCH

CD1a and CD1d Genes Polymorphisms in Breast, Colorectal and Lung Cancers

Hossein Golmoghaddam · Abdul Mohammad Pezeshki · Abbas Ghaderi · Mehrnoosh Doroudchi

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Abstract CD1 molecules might contribute to anti-tumor immune response by presentation of tumor-derived lipid and glycolipid antigens to T cells and NKT cells. Polymorphisms in CD1 genes have been suggested to modify ligand binding of CD1 molecules and thereby change the antigen presenting ability of these molecules. The aim of this study was to investigate the exon 2 polymorphisms of CD1a and CD1d in several high incident cancers in Iran. For this purpose, 201 female breast cancer patients and 207 healthy women, 64 lung cancer patients and 95 healthy individuals and 109 patients with colorectal cancer and 109 healthy controls were recruited to this study. Using PCR-SSP method, no significant correlation was found in genotype and allele frequencies of CD1a between all three studied groups and their control counterparts. Moreover, a dominant frequency of CD1d 01 (A) allele was observed in the majority of studied individuals. No significant association between the CD1 polymorphisms and prognostic factors in breast, lung and colorectal cancers was detected. Our results highlight the conserved nature of CD1 genes and may point to the immuoregulatory functions of CD1 molecules in cancer that can be exerted through fine tuning of NK, T and NKT cells.

H. Golmoghaddam · A. Ghaderi · M. Doroudchi (✉)
Department of Immunology, Medical School,
Shiraz University of Medical Sciences,
P.O. Box: 71345-45794, Shiraz, Iran
e-mail: mdoroudchi@gmail.com

A. M. Pezeshki · A. Ghaderi
Shiraz Institute for Cancer Research,
Shiraz University of Medical Sciences,
Shiraz, Iran

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Introduction

CD1 molecules are antigen presenting glycoproteins, expressed particularly on the surface of antigen-presenting cells (APCs) and play a major role in presentation of lipid and glycolipid antigens to distinct subsets of T and NKT cells [1, 2]. Based on the amino acid sequence homology, CD1 proteins are categorized into three groups; group 1 containing CD1a, b and c, group 2 containing CD1d and group 3 containing CD1e which in contrast to other CD1 molecules, is localized to late endosomal compartments [1–5]. The group 1 is known to present lipid antigens to different subsets of αβ T cells and γδ T cells [5]. However, CD1d is the antigen presenting molecule to invariant natural killer T cells (iNKT) [2–5]. CD1a, b and c are induced upon dendritic cell (DC) maturation whereas CD1d is constitutively expressed by APCs and by some epithelial cells [6]. Structurally similar to MHC class I, CD1 molecules are comprised of an α heavy chain with 3 distinct domains (α_1, α_2 and α_3) associated with a non-covalently attached light chain (β_2m). In addition, the α_1 and α_2 domains make antigen binding groove of CD1 molecules [3, 4]. However, in contrast to MHC molecules, the CD1 antigen binding groove is narrow and deeply hydrophobic which enables it to present lipid and glycolipid antigens to distinct populations of T and NKT cells [3, 4, 7]. Therefore, by presenting self and foreign antigens, CD1 molecules can regulate the balance between tolerogenic as well as autoimmune responses. Until now, most of the CD1-mediated T cell activation has been attributed to the CD1-mediated immune

responses against infection [8]. However, a few studies have suggested that CD1a and CD1d may contribute to presentation of tumor-derived lipid and glycolipid antigens to tumor-specific T and NKT cells, thus promoting anti-tumor immune responses by such cytolytic cells [5, 9]. In this regard, stage-specific expression of CD1d accompanied by CD1d-restricted NKT cell cytotoxicity in myelomonocytic leukemia is reported [10]. In addition, there are several reports on the association of improved clinical outcome in squamous cell carcinoma of the tongue, ovarian and breast cancers with the high density of $CD1a^+$ DCs in the tumoral or peritumoral areas [5, 11]. Moreover, number of CD1a+ cells in tumor sentinel lymph nodes of breast cancer patients has been reported to be associated with higher tumor grade [12]. However, other researchers did not find a significant association between the density of $CD1a^+$ cells and survival rate in breast cancer patients [13, 14]. The density of CD1a+ DCs has been shown to have a predictive value in the outcome of gastric type Barrett's metaplasia [15]. Interestingly, in rectal cancer tissue, the average density of CD83+ mature dendritic cells in tumor-associated lymphoid nodules is shown to be decreased compared to that of lymphoid nodules in normal mucosa [16]. The same decrease in the number of CD83+ (but not CD1a+) dendritic cells concomitant with the increase in the TGF-beta 1 over-expression are shown to be associated with the poor prognosis in colorectal cancer [17]. Moreover, colorectal cancer patients with high numbers of CD1a+ dendritic cells in the advancing margin of the tumor are reported to have a shorter disease-free survival [18]. Higher frequencies of immature DCs are also present in the tumor-affected lung, compared to the non-affected lung [19]. In this regard; high expression of IL-10, TGF-beta and vascular endothelial growth factor in lung tumors is suggested to be associated with the increased number of immature dendritic cells [20].

Collectively, the lipid antigen presenting function of CD1 molecules and lipid alterations during cancer development [21], suggest that CD1 molecules might have functional significance in anti-tumor immune responses.

In spite of structural similarities between MHC and CD1 molecules, a limited number of polymorphisms have been reported for CD1 genes of which some have clinical significance [22–26]. Polymorphisms in exon-2 of CD1a and CD1d genes have been associated with susceptibility to *Mycobacterium malmoense* infection, and Guillain-Barre syndrome [27–29]. Since exon-2 encodes the α_1 domain of CD1 antigen binding cleft, polymorphisms in this region may affect the anti-tumor immune response. Therefore, in this study, we investigated the frequency of polymorphisms in CD1a and CD1d genes in several high incident cancers in south of Iran and compared the results with healthy controls. We also looked into the association of tumor prognostic factors with allelic and genotypic frequencies in the patients.

Subjects and Methods

Subjects

Three groups of cancer patients and their related controls were included in our study after verbal informed consent. Group 1 consisted of 201 women with breast cancer (mean age: 46±11 years) and 207 healthy control women (mean age: 56±15 years), group 2 consisted of 64 patients with lung cancer (54 males and 10 females; mean age: 65± 10 years) and 95 healthy controls (75 males and 20 females; mean age: 57±4 years) and group 3 included 109 patients with colorectal cancer (60 males and 49 females; mean age: 54±13 years) and 109 healthy controls (69 males and 40 females; mean age: 52±11 years).

All the patients and controls in each group had the same ethnic background and were from Fars province in south of Iran. Patients were referred to the laboratory by collaborating oncologists. Cancer diagnosis was approved by clinical and pathological reports. All the control healthy individuals had no history or record of cancers and/or autoimmune diseases. The clinicopathological characteristics of the breast and colorectal cancer patients are shown in Tables 1 and 2. The clinicopathological information on the lung cancer patients was limited. Briefly, 36 of the 64 (56.25%) cases were Squamous Cell Carcinoma (SCC), 15 of the 64 (23.43%) cases were Small Cell Lung Carcinoma (SCLC), 6 of the 64 (9.38%) cases were Adenocarcinoma, and one case (1.56%) was determined to be a Carcinoid. The histological type of tumor for 6 other cases (9.38%) was not determined.

DNA Extraction

Ten mL of EDTA-treated blood samples were taken from each individual. Genomic DNA was extracted from PBMCs using salting out method as described by Miller et al. [30].

Gene Amplification

In this study, the CD1a and CD1d genes were amplified using PCR-SSP method [29]. For detection of CD1a and CD1d gene polymorphisms in exon 2, two specific primers and one common primer were used and the sequences of which are shown in Table 3. Two forward and reverse primers of β-globin gene were used as the internal control.

PCR Amplification

PCR reaction was performed in total volume of 25 μL containing 14 μL sterile distilled water (DW), 2.5 μL 10X buffer, 0.75 μL $MgCl_2$ (50 mM), 0.75 μL dNTP (10 mM) (Sinagen, Iran), 1 μL of each primers (20 pM), 1 μL DNA

Table 1 The Clinicopathological data of the studied group of breast cancer patients

	BC[a] Patients characteristics	No. (%)
Stage	0	6 (2.99)
	I	30 (14.92)
	II	134 (66.67)
	III	17 (8.46)
	IV	9 (4.47)
	ND[b]	5 (2.49)
Histological type	Infiltrating Ductal Carcinoma	155 (77.11)
	Others: Medullary, Lobular, Mixed	17 (8.46)
	ND	29 (14.43)
Tumor size (cm)	≤ 2	62 (30.85)
	2.1–5	80 (39.80)
	>5	16 (7.96)
	ND	43 (21.39)
Estrogen receptor	Pos	77 (38.31)
	Neg	65 (32.34)
	ND	59 (29.35)
Progesterone receptor	Pos	85 (42.28)
	Neg	58 (28.86)
	ND	58 (28.86)
LN[c] involvement	Pos	103 (51.24)
	Neg	81 (40.30)
	ND	17 (8.46)
Metastasis	Pos	9 (4.47)
	Neg	191 (95.03)
	ND	1 (0.50)
Age of onset	≤ 30	10 (4.98)
	>30	168 (83.58)
	ND	23 (11.44)
Menopause status	Pre-menopause	75 (37.31)
	Post-menopause	43 (21.39)
	ND	83 (41.30)
Total		201 (100)

[a] *BC* Breast cancer

[b] *ND* not determined

[c] *LN* Lymph node

Table 2 The Clinicopathological data of the studied group of colorectal cancer patients

	CRC[a] Patients characteristics	No. (%)
Histological type	Adenocarcinoma	106 (97.25)
	Leiomyosarcoma	1 (0.92)
	ND[b]	2 (1.83)
Histological grade	Well differentiated	81 (74.31)
	Moderately differentiated	25 (22.94)
	Poorly differentiated	2 (1.83)
	ND	1 (0.92)
Tumor site	Colon	92 (84.40)
	Rectum	17 (15.60)
Metastasis	Pos	4 (3.67)
	Neg	82 (75.23)
	ND	23 (21.10)
Age of onset	≤ 30	3 (2.75)
	> 30	73 (66.97)
	ND	33 (30.28)
Total		109 (100)

[a] *CRC* Colorectal Cancer

[b] *ND* not determined

(0.3 ng/ml), and 2 μL of DNA Taq polymerase (2U) (Sinagen, Iran).

For specific DNA amplification we used a touch-down PCR program. for CD1a gene amplification, it consisted of initial denaturation at 94°C for 3 min followed by three loops; loop 1 included 5 cycles consisting of 94°C for 25 s, 70°C for 60 s and 72°C for 60 s; loop 2 included 21 cycles consisting of 94°C for 25 s, 64°C for 70 s, 72°C for 70 s, and loop 3 has 5 cycles consisting of 94°C for 25 s, 55°C for 60 s, 72°C for 120 s and a final extension at 72°C for 5 min. For CD1d amplification, the PCR reaction included an initial denaturation at 94°C for 6 min followed by three loops; loop 1 included 5 cycles consisting of 94°C for 30 s, 70°C for 90 s and 72°C for 60 s; loop 2 included 26 cycles consisting of 94°C for 30 s, 63.5°C for 35 s, 72°C for 60 s, and loop 3 included 4 cycles consisting of 94°C for 30 s, 55°C for 50 s, 72°C for 60 s and a final extension at 72°C for 5 min.

The PCR products of CD1a gene and CD1d genes were then run on a 2% ethidium bromide-stained agarose gel and visualized under UV light (Figs. 1 and 2).

Statistical Analysis

The difference between the allelic and genotypic frequencies of CD1a and CD1d genes in patients and controls was analyzed by Chi-square test using SPSS 11 and Epi-info 2002 softwares. The *P* values greater than 0.05 were considered non-significant.

Results

CD1a Alleles in Breast, Lung and Colorectal Cancers

Genotype and allele frequencies of the CD1a gene are shown in Table 4. We observed that there were no significant differences in the genotype as well as the allele frequencies of CD1a polymorphism in exon-2 between the patients and controls in all three groups of cancer patients.

Table 3 The primer sequences used for amplification of CD1a and CD1d genes

	Primers	Sequences
CD1a	T622 (Isoleucine)	5′-CCTCTCTCCTTCCATgTCAT-3′
	C622 (Threonine)	5′-CCTCTCTCCTTCCATgTCAC-3′
	Common primer	5′-TTCAAACTgCATTCATgggC-3′
CD1d	A354 (Threonine)	5′-gCTTCAgAgAgCggACggT-3′
	T354 (Serine)	5′-gCTTCAgAgAgCggACggA-3′
	Common primer	5′-TgAAgTCCCgCAAAggCTTT-3′
Internal controls	β globin F	5′-ACACAACTgTgTTCACTAgC-3′
	β globin R	5′-CAACTTCATCCACgTTCACC-3′

Studying different prognostic factors such as Estrogen Receptor (ER) and Progesterone Receptor (PR) expression, tumor stage, tumor size, type of tumor, lymph node involvement and tumor metastasis in breast cancer patients showed no correlation between the genotype and allele frequencies of the CD1a gene and the prognostic factors in these patients (data not shown). We did not find any significant correlation between the CD1a genotype and allele frequencies and the tumor type or available demographical characteristics of lung cancer patients (data not shown). There was also no significant association between the CD1a genotype and allele frequencies and the related prognostic factors in the colorectal cancer patients (data not shown).

In is worth mentioning that all the patients and their controls were in Hardy-Weinberg equilibrium.

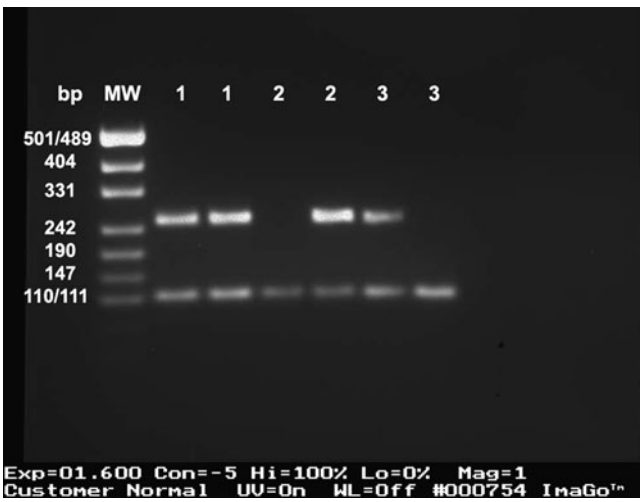

Fig. 1 Gel electrophoresis of PCR products of CD1a alleles. The result of amplification for Isoleucine/Threonine heterozygote (lanes 1 and 1), Threonine homozygote (lanes 2 and 2) genotypes and Isoleucine homozygote (lanes 3 and 3) are indicated. The reactions in both lanes 1 (Isoleucine/Threonine) indicate that the first individual is heterozygote, while lack of reaction in the first lanes 2 (Isoleucine) and the existence of PCR-amplified Threonine band in the second lane indicate that individual 2 is Threonine homozygote. The band in the first lane 3 (Isoleucine) and lack of reaction in the second lane 3 indicates that individual 3 is Isoleucine homozygote. Lane 1 = Heterozygote CT; Lane 2 = Homozygote CC; Lane 3 = Homozygote TT

CD1d Alleles in Breast, Lung and Colorectal Cancers

Analyzing the CD1d genotype frequencies in the breast cancer patients, revealed that all the patients (n=201) had AA genotype. Similarly, the frequency of AA genotype in their matched control group was 205 out of 206 (99.5%). Therefore, only one (0.5%) of the normal individuals showed a heterozygote AT genotype. As a result, the A allele with the frequency of 100% and 99.8% in the breast cancer and their related control group, respectively, dominated the frequency of the T allele.

In accordance with the finding in breast cancer patients, all the 64 lung cancer patients and their control group (n=95) were found to have the AA genotype.

Likewise, all the 109 patients with colorectal cancer and the matched 109 normal controls had AA genotype of this gene. Consequently, in all of the 784 studied individuals,

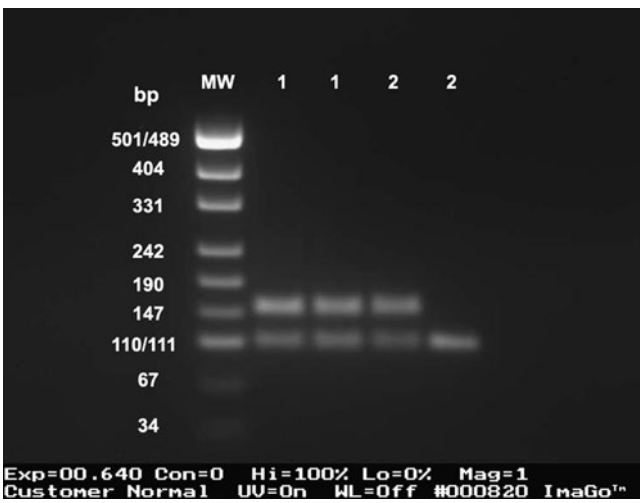

Fig. 2 Gel electrophoresis of PCR products of CD1d alleles. The result of amplification for Threonine/Serine heterozygote (lanes 1 and 1), and Threonine homozygote (lanes 2 and 2) genotypes are indicated. The reactions in both lanes 1 (Threonine/Serine) indicate that the first individual is heterozygote, while the presence of band in the first lane 2 (Threonine) and lack of reaction in the second lane (Serine) indicates that individual 2 is Threonine homozygote. Lane 1 = Heterozygote AT, Lane 2 = Homozygote AA

Table 4 Genotype and allele frequencies of CD1a gene polymorphism in Cancer patients and healthy individuals

		Patients (%)	Controls (%)	P-value
Breast cancer				
Genotypes	CC	149 (74%)	165 (80%)	0.26
	CT	46 (23%)	34 (16.5%)	
	TT	6 (3%)	7 (3.5%)	
	Total	201	206	
Alleles	C	344 (85.6%)	364 (88.3%)	0.28
	T	58 (14.4%)	48 (11.7%)	
Colorectal cancer				
Genotypes	CC	80 (73.4%)	87 (79.8%)	0.33
	CT	29 (26.6%)	20 (18.3%)	
	TT	0 (0%)	2 (1.9%)	
	Total	109	109	
Alleles	C	189 (86.7%)	194 (89%)	0.55
	T	29 (13.3%)	24 (11%)	
Lung cancer				
Genotypes	CC	48 (75%)	73 (76.8%)	0.93
	CT	12 (18.8%)	20 (21.1%)	
	TT	4 (6.2%)	2 (2.1%)	
	Total	64	95	
Alleles	C	108 (84.4%)	166 (87.3%)	0.55
	T	20 (15.6%)	24 (12.7%)	

only one AT genotype was observed in a healthy individual and all the remaining individuals had AA genotype.

Discussion

Our results showed no positive correlation between CD1a and CD1d polymorphism and susceptibility to breast, lung and colorectal cancers. To the best of our knowledge, no previous report has investigated the frequency and distribution of CD1 alleles in cancer patients. We observed a high degree of similarity between the genotype and allele frequencies of CD1a in cancer patients and healthy individuals. We also found a dominant frequency of CD1d allele 01 in all of the cancer patients as well as the control individuals. These findings underscore the high degree of conservation in CD1a and CD1d genes and is in accordance with previous reports which reported the scarcity of allele 02 in different populations [29].

In addition to having a limited number of alleles, CD1 molecules are marked by a high degree of functional conservation between human and mice [31]. Previous studies have indicated around 50% amino acid similarity in the α1 and α2 domains of human, mouse, rat and sheep CD1d molecules [32]. The considerable sequence homology between CD1 and MHC molecules especially avian MHC class I molecules has led to the conclusion that CD1 family has diverged from a common MHC ancestor gene around the time of avian-mammalian divergence [33, 34]. Considering the shared ancestral gene and the enormous difference in allelic diversification of MHC and CD1 genes, the two gene families may have evolved differentially over the past 250 million years.

It is generally accepted that conservation of a molecule over the evolutionary time is attributed to its critical functions which restricts mutational diversification of its gene [http://www.proteopedia.org/wiki/index.php/Conservation,_Evolutionary]. Several lines of evidence point to the immunoregulatory role of CD1d-dependent NKT cell mechanisms in different autoimmune diseases [35–37]. CD1d molecules are capable of presenting both self and exogenous lipids. The recognition of self antigens by NKT cells may induce the autoreactive function of NKT cells [38, 39]. It has been postulated that in the absence of foreign antigens, the autoreactive NKT cells drive monocyte differentiation towards immature DCs [39]. On the contrary, presence of infection or inflammation (for example in tumor development) can activate NKT cells in a way that these cells direct further differentiation of DCs [39]. Therefore, the fine tuning of NKT cells activity towards self lipids and glycolipids is one of the hallmarks of CD1d-dependent antigen presentation [40]. As a result, one can speculate that conserved CD1d molecules are evolutionary selected to exert their function through regulatory mechanisms rather than diversified antigen presentation. In such a scenario the presence of polymorphic alleles is not advantageous [41]. This hypothesis is well adapted with the limited antigenic diversity of microbial lipids compared to the antigenic peptides and the restricted TCR repertoire of NKT cells [41].

Similarly, CD1a and other group 1 CD1 molecules participate in the stimulation of human iDCs development from monocytes [42]. It has been shown that self-reactive T cell clones can be restricted by either of CD1a and CD1d molecules [40]. In addition, tight regulation of NK cell function by CD1a+ DCs in the human deciduas may affect successful implantation of the embryos [43]. Interestingly, there are existing evidence on the cross regulation of CD1a expression on iDCs by CD1d-dependent iNKT cell responses [44]. These data are supportive of the critical role of CD1 family in the induction and regulation of the immune response. Therefore, the limited diversification in CD1a and CD1d molecules is in line with the immuoregulatory function of these molecules and does not contradict with the observed associations with improved outcome in cancers [5, 9].

In summary, we present the first data on the CD1 gene polymorphism in cancer patients. Our results do not support any association between CD1a and CD1d alleles and

susceptibility to breast, lung and colorectal cancers. However, it does not rule out the importance of these lipid antigen presenting molecules in the immune response against cancer.

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