

Single Nucleotide Polymorphisms in Sarcomeric Protein Genes: Association with Chemotherapy Induced Cardiomyopathy

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Citation

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Abstract

Seventeen chemotherapy-induced cardiomyopathy (CM) patients and 100 volunteer blood donors (VBD) were analyzed for genetic variability in the following sarcomeric protein genes: cardiac alpha actin (ACTC); myosin light chain 2 (MYL2); myosin light chain 3 (MYL3); myosin heavy chain 7 (MYH7); myosin binding protein C3 (MYBPC3); tropomyosin 1 (TPM1); cardiac troponin I (TNNI3); and cardiac troponin T (TNNT2). No previously published mutations associated with familial CM were found in any of the subjects. No differences in the subjects and the published literature were found in the ACTC, MYL2, or TNNI3 genes. However, eight single nucleotide polymorphisms were identified in the other genes analyzed: a base change in intron 1a of TPM1; a synonymous change in exon 4 of TPM1; a base change in intron 11 of TNNT2; base changes in introns 2 and 4 of MYL3; a base change in introns 8 and 11 of MYH7; and a synonymous change in exon 31 of MYPC3. Both homozygous and heterozygous genotypes for each of these areas were identified among the subjects. Certain genotypes occurred more frequently in the CM patients and were deemed to be "skewed", with the MYH718 (TT) genotype approaching statistical significance ($p = 0.052$). Eighty-eight percent of the CM patients had one or more "skewed" genotypes, while only 64 percent of the VBD had this genetic pattern ($p = 0.032$). An increasing number of "skewed" genotypes was significantly associated with cardiomyopathy. Certain genotypes did not occur at all among the CM patients, and theoretically may confer protection against chemotherapy induced toxicity. Only one subject, a woman who developed CM on trastuzumab, had the genotype MYL312 (T/T), MYL314 (C/C), MYH7111 (CC). Additional in vitro and clinical studies are warranted to understand the phenotypic, physiologic, and clinical significances of these variabilities in the sarcomeric protein genes.

INTRODUCTION

Several antineoplastic agents (e.g. anthracyclines) used to treat human malignancies have been shown to cause clinical cardiomyopathy (CM) [1-7]. No matter their specific mechanisms of action, these agents exert their toxic effects by causing apoptosis, decreased expression of cardiomyocyte-specific genes, and/or altered molecular signaling in cardiomyocytes. Affected individuals are left with dysfunctional and fewer muscle cells available to participate in the act of contraction [1, 2, 8-11]. Despite best efforts to screen, exclude and monitor patients for left ventricular contractile problems, approximately 0.1% to 0.5% of all patients receiving cardio-toxic chemotherapy develop precipitous, clinically severe and sometimes fatal CM [1-13]. The patients literally "fall off a physiologic cliff". It is as if a certain decrement in absolute

cardiomyocytes suddenly exposes an underlying masked deficiency in cardiac contractility.

The above scenario is very similar to that of the relatively rare familial cardiomyopathies [14-22]. Approximately half of the kindreds with inherited CM have been shown to have mutations in genes encoding the sarcomeric proteins involved in the formation of the myofibrillar contractile unit in cardiomyocytes [14-22]. Changes observed include insertions, deletions, and base changes in both introns and exons and, in the latter, both synonymous and non-synonymous variations. Phenotypic penetration is variable, clinical disease development is insidious, and detection of a pre-symptomatic physiologic deficit is difficult using standard medical tests.

We reasoned that the CM observed in patients receiving

cytotoxic chemotherapy could be due to underlying low frequency mutations or single nucleotide polymorphisms (SNP) in the sarcomeric protein genes responsible for myofibril contractility. These could be due to either previously known or novel mutations. Hence, we amplified and sequenced portions of these genes from 8 CM patients and one volunteer blood donor (VBD), and subsequently examined identified regions of variability in an additional 9 CM patients and 99 VBD.

MATERIALS AND METHODS

SUBJECTS

In an IRB-approved archival study, peripheral blood samples had been obtained from 17 chemotherapy-induced CM patients (Table 1) and 100 healthy VBD. All subjects were adults (≥ 18). There was no attempt to match percentages of gender, age, race or ethnic origin between the CM patients and VBD, but approximately half were male or female and most were of European Caucasian ancestry in both groups. All 17 CM patients had normal cardiac function [as manifested by clinical history, physical exam, electrocardiogram and MUGA and/or echocardiogram assessment of left ventricular ejection fraction (LVEF)] prior to receiving chemotherapy. All had significant decreases in LVEF ($\geq 15\%$) and absolute LVEF $\leq 40\%$ after receiving chemotherapy; and all had symptoms of congestive heart failure. Five patients had acute myelogenous leukemia, 1 acute lymphocytic leukemia, 1 chronic myelogenous leukemia, 4 non-Hodgkins lymphoma, 1 Hodgkins lymphoma, 2 multiple myeloma, 1 lung cancer and 2 breast cancer. Six patients developed CM after high dose chemotherapy and autologous peripheral blood stem cell transplant, while the others had received conventional dose chemotherapy. Twelve of the patients had received an anthracycline. Patients 12 and 17 developed CM after they were treated with the anti-Her/neu2 monoclonal antibody, trastuzumab. Patient 15 had been treated with imatinib. The complete list of chemotherapy agents to which the patients were exposed, is shown in Table 1. Most of the patients responded to treatment for CM and none died from this complication.

Figure 1

Table 1. Clinical characteristics of the chemotherapy induced cardiomyopathy patients

Patient #	Gender	Diagnosis	Chemo-received	Stem Cell Tx.
1	F	myeloma	cyclophosphamide	yes
2	F	acute myelogenous, leukemia	daunorubicin, cytarabine, etoposide, mitoxantrone	yes
3	F	lymphoma	cyclophosphamide, doxorubicin, vincristine etoposide, radiation, prednisone	yes
4	M	lymphoma	cyclophosphamide, cytarabine, doxorubicin, dacarbazine, methotrexate	no
5	F	lung cancer	carboplatin, navelbine, radiation, gemcitabine	no
6	F	acute lymphocytic leukemia	cyclophosphamide, cytarabine, doxorubicin, dacarbazine, methotrexate	no
7	M	acute myelogenous, leukemia	daunorubicin, cytarabine	no
8	F	lymphoma	cyclophosphamide, doxorubicin, rituxan ifosfamide, etoposide, carboplatin, prednisone	no
9	F	acute myelogenous, leukemia	daunorubicin, cytarabine	no
10	M	myeloma	cyclophosphamide	yes
11	M	acute myelogenous, leukemia	daunorubicin, cytarabine, etoposide	yes
12	F	breast cancer	doxorubicin, trastuzumab, radiation, paclitaxel	no
13	F	Hodgkins lymphoma	radiation, nitrogen mustard, vincristine, procarbazine, adriamycin, bleomycin, vinblastine, dacarbazine	no
14	M	acute myelogenous leukemia	cytarabine, daunorubicin, etoposide	no
15	M	chronic myelogenous leukemia	imatinib	no
16	F	lymphoma	rituximab, cyclophosphamide doxorubicin, vincristine, prednisone, methotrexate, etoposide, carmustine	yes
17	F	breast cancer	paclitaxel, trastuzumab	no

PCR AND SEQUENCING

Oligonucleotides were synthesized based on sequences available online and served as primers and probes for all PCR reactions [23]. These were designed to overlap each other and encompass the exons and parts of introns of the following sarcomeric protein genes: tropomyosin 1 (TPM1); troponin I3 (TNNI3); troponin T2 (TNNT2); cardiac alpha actin (ACTC); myosin light chain 2 (MYL2); myosin light chain 3 (MYL3); myosin binding protein C (MYBPC3); and myosin heavy chain 7 (MYH7). Additional published primers and probes were used to analyze for deletions in intron 32 of the MYBPC3 gene (21). It should be noted that less than 25% of intronic DNA was analyzed in this study.

DNA was extracted from plasma or peripheral blood mononuclear cells and 0.1 μg was subjected to PCR as previously described [24]. The amplified DNA was run on gels; the desired amplified products were identified by ethidium bromide staining and confirmed by Southern blot hybridization, and the appropriate DNA cut out and eluted, as previously described [24]. The eluted DNA was re-amplified and sequenced as previously described, using the

above primers to initiate the sequencing reaction [25]. The following additional primers were synthesized to facilitate sequencing: 5'-AGA TCT TTG ACC TTC GAG GCA - 3' (TNNT2 exon7); 5'-TGC ATG TGT GTG CAG GCG TGT G - 3' (TNNT2 exon 3); and 5'- GTA AAT CAG GAA GAG AGG GCT - 3' (TNNT2 exon 11). All sequencing was done in both directions and 3 replicates were done in areas of genetic variability. If there was any confusion regarding heterozygosity, the amplified DNA was cloned and individual clones sequenced to confirm the genotype (25). The difference in the prevalence of different genotypes between the CM and VBD populations was analyzed statistically using Fisher's T-test.

RESULTS

No previously published mutations associated with familial CM were found in any of the subjects. No differences among the subjects and the published literature were found in the ACTC, MYL2 or TNNT3 genes. However, eight changes were found in the other genes analyzed: a change (TTC) at base 2,129 in intron 1a (TPM1I1a) and a synonymous change (CAA) at base 17,868 in exon 4 (TPM1E4) of the TPM1 gene; a change (CAA) at base 11,643 in intron 11 of the TNNT2 gene (TNNT2I11); a change (GTT) at base 1224 in intron 2 (MYL3I2) and a change (AAC) at base 3800 in intron 4 (MYL3I4) of the MYL3 gene; a change (CTT) at base 7,863 in intron 8 (MYH7I8), and a change (TTC) at base 8,741 in intron 11 (MYH7I11) of the MYH7 gene; and a synonymous change (AAG) at base 20,342 in exon 31 (MYBPC3E31) of MYBPC3 gene. All of the changes observed in introns occurred within 50 bases downstream from a splice site. Hence, there were eight previously undefined regions of SNP identified in the sarcomeric protein genes analyzed. Homozygous and heterozygous subjects were identified; the prevalence of the various genotypes in the CM and VBD populations are shown in Table 2 and the genotypes of each CM patient are shown in Table 3. CM patient 12 is the only person found to have the TT genotype for MYL3I2, the CC genotype for MYL3I4, and/or the CC genotype for MYH7I11 among all 117 subjects. The difference in prevalence of each of these three genotypes between the CM and VBD populations does not reach statistical significance (p = 0.145), but does if one compares the prevalence of all three occurring simultaneously among the total MYL3 and MYH7I11 genotypes analyzed (p = 0.003). Interestingly, this is one of two subjects who developed their CM while on the anti-HER/neu 2 monoclonal antibody trastuzumab. The only

other unique genotype identified in CM patients was the heterozygous CT genotype in TPM1I1a found in patient 5. Interestingly, the homozygous CC genotype for TPM1I1a was not observed among any of the 117 subjects analyzed, suggesting it is either very rare or deleterious.

Figure 2

Table 2. Prevalence of different sarcomeric protein genotypes among chemotherapy - induced cardiomyopathy (CM) patients and volunteer blood donors (VBD)

Subject type	Genotypes for Indicated Gene Site *																							
	TPM1I1a		TPM1E4		TNNT2I11		MYL3I2		MYL3I4		MYH7I8		MYH7I11		MYBPC3E31									
	TT	CT	CC	CC	CA	AA	CC	CA	AA	GG	GT	TT	AA	CA	CC	CC	CT	TT	TT	CT	CC	AA	AG	GG
CM	16	1	0	4	5	8	6	8	3	14	2	1	15	1	1	11	4	2	13	3	1	1	7	9
VBD	100	0	0	19	43	38	52	43	5	77	23	0	97	3	0	68	31	1	85	15	0	15	45	40

* frequencies in the boxed genotypes are skewed toward cardiomyopathy patients

+ p values for skewed vs non-skewed genotypes: TPM1I1a p = 0.145; TPM1E4 p = 0.162; TNNT2I11 p = 0.075; MYL3I2 p = 0.145; MYL3I4 p = 0.145; MYH7I8 p = 0.052; MYH7I11 p = 0.145; MYBPC3E31 p = 0.127

Figure 3

Table 3: Genotypes of each cardiomyopathy patient relative to sarcomeric protein SNPs

Subject	Genotypes for Indicated Gene Site														Total # of skewed genotypes								
	TPM1I1a		TPM1E4		TNNT2I11		MYL3I2		MYL3I4		MYH7I8		MYH7I11			MYBPC3E31							
	TT	CT	CC	CC	CA	AA	GG	GT	TT	AA	CA	CC	CC	CT		TT	TT	CT	CC	AA	AG	GG	
P-1	TT			CC		CA		GT		CA		CC		TT							GG		1
P-2	TT			AA		CA		GG		AA		CC		TT							AG		1
P-3	TT			AA		CA		GG		AA		CT		TT							AG		1
P-4	TT			AA		CA		GG		AA		CT		CT							GG		2
P-5	CT			AA		CA		GG		AA		CC		TT							GG		3
P-6	TT			AA		CC		GG		AA		CC		TT							AG		1
P-7	TT			AA		AA		GG		AA		CC		TT							GG		3
P-8	TT			AA		AA		GG		AA		CC		TT							GG		2
P-9	TT			CC		CA		GG		AA		CT		CT							AG		0
P-10	TT			CA		CA		GG		AA		CC		TT		AA					GG		0
P-11	TT			CA		CA		GG		AA		CC		TT							GG		1
P-12	TT			CA		CC		GG	TT		CC		TT		CC						AG		4
P-13	TT			CA		AA		GG		AA		CC		TT		CC					AG		1
P-14	TT			AA		CC		GT		AA		CC		TT							GG		2
P-15	TT			CC		CC		GG		AA		CC		TT							GG		2
P-16	TT			AA		CC		GG		AA		CC		TT							AG		1
P-17	TT			CA		CA		GG		AA		CC		CT							GG		1

+ = genotypes skewed to cardiomyopathy patients

Certain genotypes occurred with greater frequencies in the CM population (Table 2). If genotypes that occurred with at least a ≥6% greater absolute frequency in the CM population compared to the VBD, are labeled as “skewed” and those that did not as “non-skewed”, several themes emerge. First, the differences in the “skewed” and “non-skewed” genotypes for TNNT2I11 (AA vs. CC or CA) and MYH7I8 (TT vs CC or CT) do approach statistical significance (p = 0.075 and 0.052, respectively) (Table 2). Second, 15 out of 17 of the CM patients had one or more “skewed” genotypes while 36 of the VBD did not (p = 0.032) (Table 4). The statistical significance increases when the differences in higher numbers of “skewed” genotypes are compared (Tables 5 and 6).

Figure 4

Table 4: Distribution of total skewed genotypes among cardiomyopathy patients and volunteer blood donors

subject type	total # (%) of skewed genotypes	
	0	1-4
C M	2 (12)	15 (88)
VBD	36 (36)	64 (64)

p = 0.032

Figure 5

Table 5: Distribution of total skewed genotypes among cardiomyopathy patients and volunteer blood donors

subject type	total # (%) of skewed genotypes	
	0-1	2-4
C M	10 (59)	7 (41)
VBD	84 (84)	16 (16)

p = 0.024

Figure 6

Table 6: Distribution of total skewed genotypes among cardiomyopathy patients and volunteer blood donors

subject type	total # (%) of skewed genotypes	
	0-2	3-4
CM	14 (82)	3 (18)
VBD	100 (100)	0 (0)

p = 0.003

DISCUSSION

The 17 chemotherapy induced CM patients studied, herein, were identified over a multiyear period and were found among at least a thousand patients treated with cardiotoxic chemotherapy, an anticipated prevalence rate. Because the development of CM occurs so infrequently among patients treated with chemotherapy, the VBD population would predictably reflect the genotypic profile of patients treated with chemotherapy who did not develop CM.

Obviously, there could be many reasons that could explain differences in susceptibility to the cardio-toxic effects of chemotherapy. These could include differences in pharmacokinetics, drug metabolism or activation and intracellular sensitivity to deleterious effects. We have chosen to study whether genetic alterations to sarcomeric protein genes could result in either different levels of protein expression or mutant proteins that would decrease the inherent contractibility of a patient's cardiomyocyte, thereby, leading to decreased tolerance of a particular degree of cardiomyocyte injury.

While we did not necessarily find a specific mutation that would predict for all chemotherapy induced CM, we did identify a total of eight SNPs in the introns and exons of five sarcomeric protein genes. Various genotypes defined by these SNPs were "skewed" toward the CM population and some to a degree that approached statistical significance. The CM patients had a statistically significant higher number of these "skewed" genotypes compared to VBD.

It is important to note that synonymous mutations can lead to different phenotypic expression due to several mechanisms, the most common being different levels of transfer RNAs for the different codons that are translated into the same amino acid [26]. Also, mutations in introns within up to 100 bases of a splice site can effect RNA splicing efficiency, resulting in variable protein expression [26].

We plan several investigations relative to this project. First, we will analyze the 17 CM and 100 VBD DNAs for genetic changes in the remaining intronic DNA contained in the eight genes studied, herein, and for variations among other remaining cardiac sarcomeric protein genes associated with the contractile unit, eg titan and tropomyosin 2. Second, we will analyze additional CM samples to ascertain the prevalence rate of the "skewed" genotypes identified in the CM population, particularly those which already approach statistically significant differences from the VBD. Obviously, the MYL3I2 = TT, MYL3I4 = CC and MYH7I11 = CC genotype needs to be further characterized in the CM population, particularly where trastuzumab is felt to be the cause of cardiomyopathy. Further, each of the skewed genotypes should be examined in different chemotherapy subsets, eg conventional vs high dose chemotherapy and other forms of cardiac injury such as ischemia or HIV infection etc. We also plan to investigate whether the putative "protective" genotypes identified would predict for superior cardiac function in athletes or following other cardiac injuries, such as ischemia or HIV infection, etc. Finally, we also plan studies on clinical samples, animal models, and in vitro systems, wherein sarcomeric genotypes will be correlated with their cognate protein expression and concomitant cardiomyocyte function. We anticipate such studies will further clarify the genetic determinants of cardiac function in health and disease.

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