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Polymorphic Variations Associated With Doxorubicin-Induced Cardiotoxicity in Breast Cancer Patients

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Doxorubicin (DOX) is a commonly used antineoplastic agent for the treatment of various malignancies, and its use is associated with unpredictable cardiotoxicity. Susceptibility to DOX cardiotoxicity is largely patient dependent, suggesting genetic predisposition. We have previously found that individual sensitivity to DOX cardiotoxicity was associated with differential expression of genes implicated in inflammatory response and immune trafficking, which was consistent with the increasing number of reports highlighting the important role of human leukocyte antigen (HLA) complex polymorphism in hypersensitivity to drug toxicity. This pilot study aimed to investigate DNA from patients treated with DOX-based chemotherapy for breast cancer and to correlate the results with the risk for DOX-associated cardiotoxicity. We have identified 18 SNPs in nine genes in the HLA region (NFKBIL1, TNF-α, ATP6V1G2-DDX39B, MSH5, MICA, LTA, BAT1, and NOTCH4) and in the psoriasis susceptibility region of HLA-C as potential candidates for association with DOX cardiotoxicity. These results, albeit preliminary and involving a small number of patients, are consistent with reports showing the presence of susceptibility loci within the HLA gene region for several inflammatory and autoimmune diseases, and with our previous findings indicating that the increased sensitivity to DOX cardiotoxicity was associated with dysregulation of genes implicated both in inflammation and autoimmune disorders.

Key words: Doxorubicin (DOX); Cardiotoxicity; Genotyping; Breast cancer

INTRODUCTION

Doxorubicin (DOX) is a commonly used anthracycline anticancer agent for the treatment of various malignancies, and it may cause unpredictable cardiotoxicity[1]. The mechanism(s) of DOX cardiotoxicity is still uncertain; however, it is likely a multifactorial event involving diverse processes such as oxidative stress, inhibition of nucleic acid and protein synthesis, release of vasoactive amines, abnormalities in Ca^{2+} handling, activation of the ubiquitin–proteasome system, and impaired cardiac repair due to inhibition of bone marrow and cardiac progenitor cells[2].

It has been well established that DOX cardiotoxicity is a cumulative dose-dependent process that begins with the first dose, suggesting that assessment of the cardiac function in patients prior to chemotherapy may be able to avoid permanent cardiac damage[3]. According to the American College of Cardiology guidelines, patients receiving chemotherapy are at an increased risk of developing cardiac dysfunction[4]. Evidence indicates that susceptibility to DOX cardiotoxicity is largely individual, with some patients developing cardiomyopathy at doses of 200–400 mg/m^{2}[3], and others tolerating much higher cumulative doses up to >800–1,000 mg/m^{2}[5], suggesting the presence of a genetic predisposition. Genetic variations in ABCB1, SLC22A16, and CBR1 genes were suggested to contribute to DOX adverse effects[6]. We have previously found that individual sensitivity to a low dose of DOX-based chemotherapy was associated with differential expression of genes implicated in inflammatory response and immune trafficking[7], which was consistent with the increasing number of reports showing the important role of human leukocyte antigen (HLA) complex polymorphism in hypersensitivity to drug toxicity[8].

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This pilot study focused on obtaining further information about the genetic variations of cancer patients who develop cardiac abnormality after DOX-based chemotherapy and compare them with patients who maintain normal cardiac function.

MATERIALS AND METHODS

Ethical Statements

This study was carried out in accordance with the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the ethics committee of the University of Arkansas for Medical Sciences. All subjects signed an Institutional Review Board-approved informed consent where they were informed about the use of their blood samples and medical records for research purposes.

Patients

Thirty women were treated for breast cancer at the University of Arkansas for Medical Sciences between 2011 and 2015, and they were enrolled in an institutional review board-approved protocol and gave informed consent. The disease stage of all patients is presented in Table 1 under TNM stage. Sixteen patients were diagnosed with stage IIA stage disease, of which six patients had an abnormal decline of LVEF at completion of chemotherapy; eight patients had stage IIB breast cancer, of whom two developed abnormal LVEF decline; five patients had stage IIIA disease, of whom one developed abnormal LVEF decline; and one patient with abnormal LVEF had stage IIIC disease. All patients were treated with a combination of DOX (60 mg/m^2) with cyclophosphamide

Table 1. Patient Demographics, Tumor Characteristics, Comorbid Conditions, and Concomitant Medications

Characteristic	All Patients	Patients With Abnormal Decline of LVEF
Patients analyzed	30	10
Race		
European American	21	9
African American	9	1
Age (years)		
Median	53.1	57.6
Range	35–76	47–66
Tumor type: ductal	30	10
Tumor grade		
I	6	2
II	13	6
III	11	2
TNM stage		
IIA	16	6
IIB	8	2
IIIA	5	1
IIIC	1	1
Hormone receptor status		
ER$^+$, PR$^+$/Her2-neu	20	6
ER$^-$, PR$^-$	9	
ER$^-$/PR$^+$	1	1
HER2$^+$	1	1
Triple negative	9	2
Lymph node positive	20	6
Comorbidity and medications		
Hypertension	9	4
Hydrochlorothiazide, 12.5 mg; metoprolol, 25 mg; candesartan, 16 mg; nebivolol, 10 mg; amlodipine, 10 mg; valsartan, 12.5 mg; lisinopril, 10 mg		
Diabetes mellitus	6	3
Metformin, 1,000 mg; insulin glargine, 100 U/ml		
Coronary artery disease	1	1
Atenolol, 25 mg		
Autoimmune diseases		
Asthma	1	
Albuterol; mometasone		

(600 mg/m^2) every 3 weeks for four cycles (cumulative dose of DOX 240 mg/m^2 and cumulative dose of cyclophosphamide 2,400 mg/m^2). Cardiac function of all subjects was assessed by multigated acquisition (MUGA) scan before the start of the DOX-containing chemotherapy and after the completion of DOX-based chemotherapy. A decline of LVEF by >10% or below 55% was considered abnormal.

Methods and Data Analysis

DNA was extracted from peripheral blood using QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA, USA). Samples were quality assessed and quantified by ultraviolet (UV) absorbance measured via the NanoDrop Technologies NanoDrop® ND-2000 Spectrophotometer (Wilmington, DE, USA) and software. DNA quality was evaluated by an allelic discrimination drug-metabolizing enzyme assay (Applied Biosystems Assay; Life Technologies, Carlsbad, CA, USA) with fluorescence detection on the 7900 Fast Real-Time PCR System (Applied Biosystems, Life Technologies) performed according to the manufacturer's protocol. Genotyping was performed using the Illumina® HumanOmni5-4v1.1 BeadChip. Beadchips were imaged using the Illumina iScan System and analyzed with GenomeStudio V20111 software (Illumina), which has a built-in cnvPartition tool for copy number variants (CNVs). For whole-genome SNP genotyping analysis, individual samples with genotype call rates <93% and SNPs with call rates <95% were removed. The Cochran–Armitage test for trend was conducted with Purcell's PLINK program (http://pngu.mgh.harvard.edu/purcell/plink). To map the SNPs-to-genes, containing genomic coordinates for all genes according to positions on the Genome Browser, hg18 (NCBI assembly GRCh36) were downloaded from the PLINK ftp server (http://pngu.mgh.harvard.edu/~purcell/plink/res.shtml#hapmap) accessed in January 2015. SNPs were assigned to a gene if they located within its primary transcript (intragenic region) or 5 kilobases (kb) upstream or downstream of the gene start or end[9]. The small sample size precluded the ability to detect genome-wide associations; therefore, we used a candidate gene/SNP approach for analysis. We used a 5% level of significance criteria to identify the significant SNPs at first hand. Based on previous data showing significant alterations in the gene expression of genes associated with inflammation and immunity in patients with DOX-associated abnormal decline of LVEF, we manually selected SNPs ($p<0.05$) that have been reported in autoimmune and inflammatory diseases.

For CNV analysis, the algorithm parameters in cnvPartition 2.4.4 were set at 25 minimum probes and 35 minimum confidence value threshold for CNV detection, and 250-kb minimal region size for LOH detection. Log R ratios (LRR) and B allele frequencies (BAF) were visualized for every CNV and CN-LOH call on each chromosome via Genome Studio's chromosome browser. Each copy number event was assigned one of four possible calls based on the BAF, LRR, copy number values, and confidence scores: (1) LOH (BAF≠0.5 and split into two components; LRR<0; CNV=1; confidence>200), (2) homozygous deletion (BAF=SNPs scattered between 0 and 1; LRR<−1; CNV=0; confidence>200), (3) duplication (BAF split 0.65/0.35; LRR>0; CNV>2; confidence>200), (4) CN-LOH (BAF split into two components, normally 0.95/0.5; LRR=0; CNV=2; confidence>200; FISH negative). Long stretches of genomic DNA without CNAs have BAF=0.5, LRR=0, and CNV=2. The mapping of chromosomal regions to genes was performed using the gene definitions and coordinates from UCSC Genome Build hg18 (the refFLAT file downloaded from UCSC database).

RESULTS AND DISCUSSION

We have analyzed and compared the genotype distribution of two distinct groups of breast cancer patients treated with DOX-based chemotherapy: 10 patients who developed abnormal LVEF decline at the completion of chemotherapy and 20 patients who did not. Patients' demographics, tumor characteristics, comorbid conditions, and concomitant medications are presented in Table 1. All 30 patients were diagnosed with invasive ductal carcinoma (IDC). In this patient population, 9 patients were African American (AA) and 21 were European Americans (EA). One of the AA patients and nine of the EA patients presented with abnormal decline of LVEF at completion of chemotherapy. The patients with abnormal decline of LVEF were in the age range of 47–66 years, with mostly stage IIA disease ($n=6$) and hormone receptor status mainly ER$^+$/PR$^+$ and Her2-neu$^+$. Nine of the patients had hypertension; of these, three presented with abnormal LVEF decline, three of the patients with abnormal LVEF had diabetes (3:6), one had coronary artery disease (1:1), and one patient with normal LVEF had asthma. Table 2 presents the average changes of LVEF of the patients with abnormal decline of LVEF at completion of chemotherapy and patients who maintained normal LVEF. These results may suggest the possible involvement of hypertension and diabetes in the increased sensitivity to DOX cardiotoxicity, but the small number of patients does not allow definitive conclusions.

A total of 1,859 autosomal SNPs passed quality thresholds ($p<0.05$) for association analysis. Potential candidate genes in chromosome 6p32 and 6p33 were identified (Table 3). These included 15 SNPs in nine genes in the HLA region (NFKBIL1, TNF-α, ATP6V1G2-DDX39B, MSH5, MICA, LTA, BAT1, NOTCH4), and 3 SNPs in the psoriasis susceptibility region of HLA-C (rs9264942, rs2523619, and rs10484554) (Table 1). Six of the SNPs in the BAT1-NFKBIL1-LTA region (rs2071591, rs3093949,

Table 2. Cardiac Function of Women With Breast Cancer, Treated With DOX-Based Chemotherapy

	No. of Patients	Average LVEF (%) at Baseline (Mean ± SD)	Average LVEF (%) at Completion of Chemotherapy (Mean ± SD)
Patients with abnormal LVEF (%)	10	69.875 ± 5.436	57.875 ± 3.833
18–47 years	2	68.5 ± 2.121	57.5 ± 0.707
48–55 years	2	64.0 ± 0	54.0 ± 0
>56 years	4	73.5 ± 5.066	60.0 ± 4.242
Patients with normal LVEF (%)	20	61.583 ± 5.523	61.833 ± 6.919
18–47 years	9	60.888 ± 6.050	62.222 ± 9.162
48–55 years	9	60.888 ± 5.065	59.777 ± 5.118
>56 years	7	63.142 ± 5.080	57.714 ± 6.499

MUGA scan was performed before the start of DOX chemotherapy and at its completion. LVEF, left ventricle ejection fraction.

rs2071592, rs2239527, rs909253, and rs1041981) have been reported in association with various inflammatory and autoimmune disorders including rheumatoid arthritis (RA)[10], myocardial infarction[11], and Grave's disease[12]. TNF-α rs1800629 polymorphism was reported to be an associated risk for coronary heart disease and myocardial infarction[13] and Crohn's disease[14]. C6orf10 rs2050190 was associated with coronary artery disease[15], MSH5 rs3131379 and rs3131378 in systemic lupus erythematosus (SLE)[16], and MICA rs2523451 was reported as a marker for RA[17]. NOTCH4 rs3134931 was identified in coronary artery diseases[18]. HLA-C rs9264942, rs2523619, and rs10484554 were associated with psoriasis and psoriatic arthritis[19], and rs9264942 was reported in patients with inflammatory bowel disease[20]. The telomeric class III region of HLA bordering the class I region is particularly gene dense, containing at least 10 genes in addition to TNF within an 82-kb interval: BAT1, ATP6V1G2, NFkBIL1, LTA, TNF, LTB, LST1, NCR3, AIF-1, BAT3, and BAT2[21]. The function of most of these molecules remains poorly characterized, although evidence suggests their role in immune and inflammatory responses does exist for several[22]. NF-kB, a transcription factor that modulates the transcription of a variety of genes, including cytokines and growth factors, adhesion molecules, immune receptors, and acute phase proteins, is considered an important factor in inflammation and has been implicated in RA, atherosclerosis, asthma, multiple sclerosis (MS), inflammatory bowel disease, and ulcerative colitis[23].

Table 3. SNPs in chr6p Associated With Abnormal Decline of LVEF in Patients Treated With DOX-Based Chemotherapy

SNP	Gene Symbol	Base Position	Minor Allele	Major Allele	Min	Maj	All	Abnormal LVEF	Normal LVEF	OR	*p* Value
rs9264942	HLA-C	31274380	G	A	0.6	0.15	4/10/16	4/10/16	0/6/14	8.61	0.01
rs2523619	HLA-C	31318144	G	A	0.55	0.15	4/9/17	4/9/17	0/6/14	6.56	0.01
rs10484554	HLA-C	31274555	A	G	0.35	0.07	2/6/22	2/6/22	0/3/17	5.41	0.04
rs2071591	NFKBIL1	31515799	A	G	0.6	0.25	5/12/13	5/12/13	1/8/11	6.83	0.02
rs3093949	NFKBIL1	31525184	A	G	0.6	0.22	5/11/14	5/11/14	1/7/12	8.87	0.01
rs2071592	NFKBIL1	31515340	A	T	0.6	0.22	5/11/14	5/11/14	1/7/12	7.99	0.01
rs2071594	ATP6V1G	31512720	C	G	0.6	0.25	5/12/13	5/12/13	1/8/11	6.83	0.02
rs3130059	ATP6V1G	31509284	G	C	0.6	0.25	5/12/13	5/12/13	1/8/11	6.83	0.02
rs11796	ATP6V1G	31501212	T	A	0.6	0.27	6/11/13	6/11/13	2/7/11	4.12	0.03
rs2050190	C6orf10	32339076	G	A	0.6	0.2	6/8/16	6/8/16	1/6/13	3.89	0.02
rs1800629	TNF-α	31543031	A	G	0.35	0.07	2/6/22	2/6/22	0/3/17	5.67	0.03
rs3131379	MSH5	31721033	A	G	0.2	0.05	0/6/24	0/6/24	0/2/18	11.58	0.04
rs3131378	MSH5	31725285	G	A	0.2	0.05	0/6/24	0/6/24	0/2/18	11.58	0.04
rs2523451	MICA	31369151	A	G	0.5	0.22	3/13/14	3/13/14	0/9/11	4.50	0.04
rs909253	LTA	31540313	G	A	0.6	0.25	5/12/13	5/12/13	1/8/11	6.83	0.02
rs1041981	LTA	31540784	A	C	0.6	0.25	5/12/13	5/12/13	1/8/11	6.83	0.02
rs2239527	BAT1	31509779	G	C	0.5	0.25	4/12/14	4/12/14	1/8/11	4.13	0.05
rs3134931	NOTCH4	32190620	G	A	0.2	0.45	3/16/11	3/16/11	3/12/5	0.22	0.05

Min, minor allele frequency; Maj, major allele frequency; All, distribution of genotypes among all subjects treated with Dox-based chemotherapy (genotypes are ordered as mm/Mm/MM, where "m" and "M" are the minor and major alleles, respectively); abnormal LVEF, distribution of genotypes among subjects who developed abnormal LVEF decline; normal LVEF, distribution of genotypes among subjects who maintained normal LVEF; OR, estimated odds ratio for development of DOX cardiotoxicity (for minor allele; major allele is a reference).

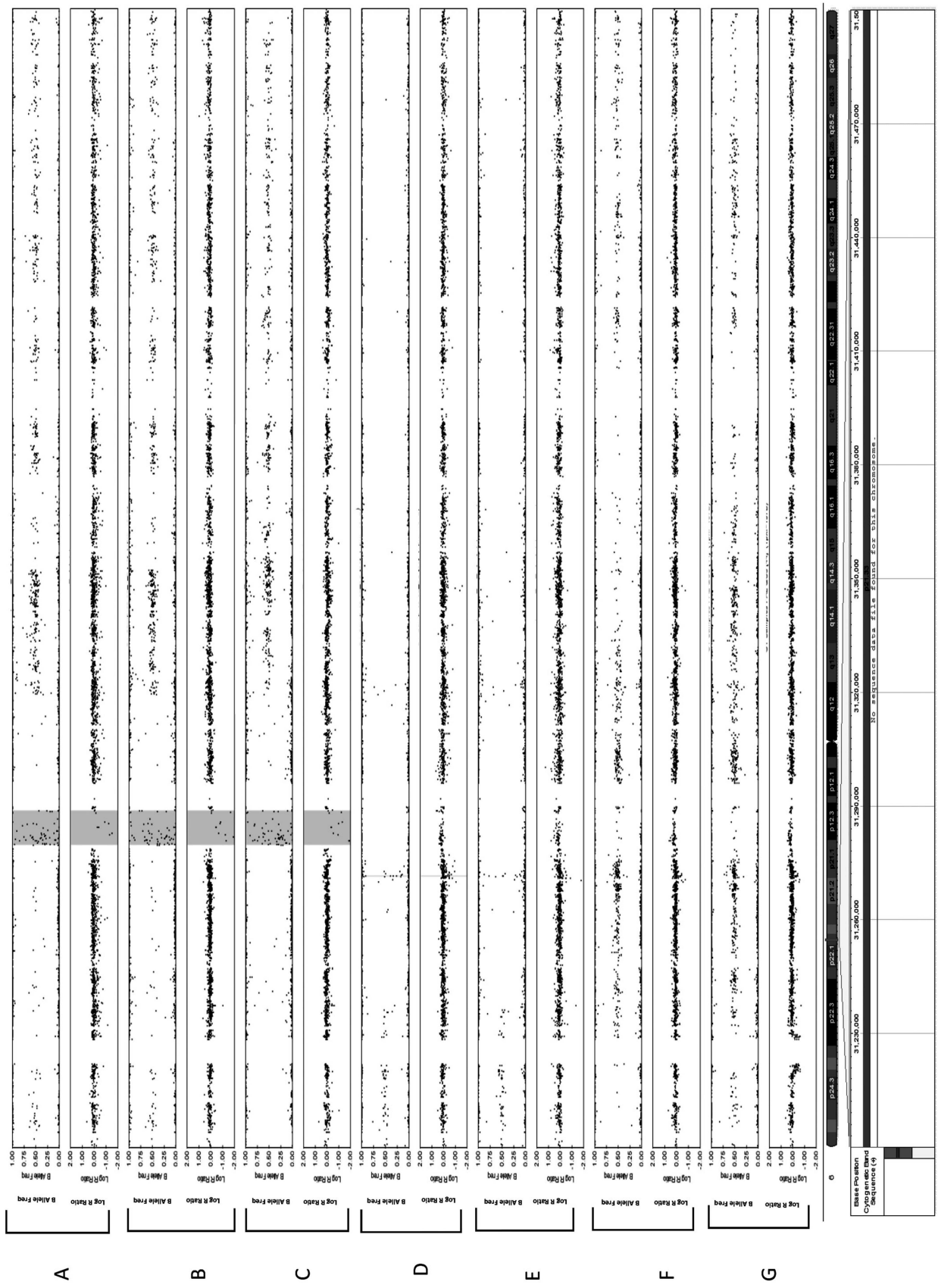

Figure 1. Homozygous deletion in chr6p.32 in the area of HLA-B.

The CNV analysis identified a total of 311 CNVs among the patients in both examined groups, with abnormal and normal LVEF. Most of the CNVs were loss of heterozygosity (LOH) and duplications, without correlation with the heart function. Three of the patients with abnormal decline of LVEF showed LOH of 22 kb in the region of the HLA-DRB5 gene (chr6p.32), and three other patients with abnormal decline of LVEF showed homozygous deletion of 9 kb in chr6p.32 in the area of HLA-B (Fig. 1).

In conclusion, our data, although preliminary and involving a small number of cancer patients, are consistent with reports that have identified the presence of susceptibility loci within the HLA gene region for several inflammatory and autoimmune diseases, including RA, SLE, MS, and Grave's diseases[24]. Autoimmune features and rheumatic manifestations have been reported in cancer patients after chemotherapy[25], as well as the association between chemotherapy and development of rheumatism, and SLE has been reported in breast cancer[26], although the mechanism of this syndrome was not established. In addition, evidence indicates that systemic inflammatory diseases are associated with increased coronary artery disease morbidity and mortality[27]. Moreover, our data are in line with our previous findings showing that the increased susceptibility to DOX cardiotoxicity was associated with dysregulation of genes implicated both in inflammation and immunity[5]. To confirm our finding, a targeted SNPs study is warranted.

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