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Whole exome sequencing to identify genetic markers for trastuzumab-induced cardiotoxicity

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Although trastuzumab-induced cardiotoxicity is an important determinant to limit the use of this drug, the molecular mechanism of risk for this toxicity is not well understood. To identify genetic variants determining the risk of trastuzumab-induced cardiotoxicity, we carried out whole exome sequencing of germline DNA samples from 9 patients with trastuzumab-induced cardiotoxicity, and conducted a case-control association study of 2258 genetic variants between 9 cases (with trastuzumab-induced cardiotoxicity) and general Japanese population controls registered in the Human Genetic Variation Database (HGVD). The top variant which showed the lowest *P*-value in the screening study was rs139503277 in PHD Finger Protein 3 (P_{min} = .00012, odds ratio [OR] = 51.23). To further validate the result of screening study, we carried out a replication study of 10 variants showing $P_{min} < .001$ in the screening study using 234 independent patients treated with trastuzumab, including 10 cases and 224 controls (without trastuzumab-induced cardiotoxicity). In the replication study, we observed that three variants had an effect in the same direction as in the screening study (rs78272919 in exon 2 of Keratin 15, rs5762940 in exon 2 of zinc and ring finger 3, and rs139944387 in exon 44 of Eyes shut homologs [*EYS*]). A combined result of the screening and the replication studies suggested an association of a locus on chromosome 6q12 with trastuzumab-induced cardiotoxicity (rs139944387 in *EYS*, combined P_{min} = .00056, OR = 13.73). This finding provides new insights into personalized trastuzumab therapy for patients with human epidermal growth factor receptor 2 (HER2)-positive cancer.

KEYWORDS
cardiotoxicity, HER2, pharmacogenomics, trastuzumab, whole exome sequencing

1 | INTRODUCTION

Trastuzumab (Herceptin) is a humanized monoclonal antibody that targets HER2.[1] HER2 is a transmembrane protein, a member of the EGFR family and is overexpressed or amplified in approximately 20%-30% of breast cancer patients.[2,3] In HER2-overexpressing

Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; CI, confidence interval; EGFR, epidermal growth factor receptor; FFPE, formalin-fixed paraffin-embedded; GATK, genome analysis toolkit; gVCF, genomic variant call format; HER2, human EGFR 2; HGVD, human genetic variation database; LVEF, left ventricular ejection fraction; MAF, minor allele frequency; NCC, National Cancer Center; OR, odds ratio; SNV/indel, single nucleotide variant/insertion/deletion.

tumor cells, HER2 signaling pathways (PI3K/Akt pathway and ERK-MAPK pathway), which are involved in cell proliferation and invasive capacity, are activated.[4,5] Patients with HER2-positive breast cancer generally have a less favorable response to traditional chemotherapy and increased risk of relapse compared with those who are HER2 negative.[6] Trastuzumab binds to the extracellular domain of HER2, prevents the activation of the HER2 signaling pathway and induces ADCC.[7-10] Trastuzumab is used for treatment in HER2-positive breast or gastric cancer patients.[11-13] Chemotherapy plus first-line trastuzumab resulted in a significantly higher response rate than chemotherapy alone, and prolonged survival of patients with cancer.[14] However, it is reported in many studies that approximately 5% of patients treated with trastuzumab alone suffer cardiac dysfunction.[14-17] This toxicity is often characterized by an asymptomatic reduction in LVEF.[18,19] Although the mechanism of trastuzumab-induced cardiotoxicity is not well understood, blocking of HER2 signaling in cardiomyocytes has been suggested to be involved in this toxicity.[20,21] Anthracycline-induced cardiotoxicity is cumulative, dose-dependent, irreversible and associated with pathological changes, whereas trastuzumab-induced cardiotoxicity is largely reversible and dose-independent, which suggests that genetic factors play important roles in this adverse event.[22,23] It is reported that common polymorphisms Pro 1170 Ala and Ile 655 Val in Erb-B2 Receptor Tyrosine Kinase 2 (*ERBB2*), which encodes HER2, are associated with trastuzumab-induced cardiotoxicity;[24,25] however, associations of these polymorphisms in the candidate gene have not yet been sufficiently validated.

As trastuzumab-induced cardiotoxicity is not a common adverse event, we hypothesized that rare genetic variants, as well as common variants, could impact on the occurrence of the adverse events. In the present study, to identify genetic markers determining the risk of trastuzumab-induced cardiotoxicity, we carried out whole exome sequencing of germline DNA samples from 9 patients with trastuzumab-induced cardiotoxicity, and identified variants that were likely to be associated with risk of trastuzumab-induced cardiotoxicity.

2 | MATERIALS AND METHODS

2.1 | Patients

A total of 9 breast cancer patients with trastuzumab-induced cardiotoxicity were used for whole exome sequencing in the screening study. Germline DNA was extracted from FFPE lymph nodes which did not contain cancer cells or blood samples at the NCC (Tokyo, Japan). In the replication study, 234 patients (10 cases and 224 controls) treated with trastuzumab (breast cancer, 166 cases; gastric cancer, 64 cases; others and unknown, 4 cases) were used from the samples registered in NCC Biobank (http://www.ncc.go.jp/jp/biobank/). We defined trastuzumab-induced cardiotoxicity as that which causes ≥10% decrease of LVEF compared to before trastuzumab treatment. This study was approved by the Institutional Review Board of the NCC (Tokyo, Japan).

2.2 | Whole exome sequencing

Whole exome sequencing of DNA samples from 9 patients was carried out. Sequencing libraries were prepared using the SureSelect XT Target Enrichment Kit (Agilent Technologies, Santa Clara, CA, USA) and captured using a SureSelect Human All Exon V5+lncRNA kit (Agilent Technologies) according to the manufacturer's protocols. Each captured library was then loaded onto a HiSeq 2000 sequencing platform (Illumina, San Diego, CA, USA) with 100 base paired-end reads. Genotyping to validate the result of the 10 candidate variants that are possibly associated with trastuzumab-induced cardiotoxicity was done by Sanger sequencing or TaqMan Genotyping Assays (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

2.3 | Variant annotation and filtering

All reads were aligned to the human genome (GRCh37/hg19) with an alignment tool, BWAMEM.[26] The following reads were removed by an in-house program: (i) low mapping-quality reads; (ii) PCR duplication reads; (iii) low alignment score (using the "AS" tag of the SAM format) and mismatch rate (a percentage of the number of mismatches to length of read) ≥5% reads; (iv) high sub-optimal alignment score (using the "XS" tag of the SAM format) and mismatch rate ≥5% reads. Germline SNV/indel call's analysis pipeline was based on GATK v3.5 best practices (Broad Institute of Harvard and MIT, Cambridge, MA, USA). After running indel realignment and base quality score recalibration by IndelRealigner and BaseRecalibrator from GATK, HaplotypeCaller was used to create gVCF for each sample. The gVCF files of each sample were merged by GenotypeGVCF. The following calls were filtered by the VariantFiltration. (i) SNV: SnpCluster (clusterSize = 3, clusterWindowSize = 10), quality by depth (QD <2.0), Phred-scaled *P*-value using Fisher's exact test to detect strand bias (FS >60.0), mapping quality (MQ <40.0), MappingQualityRankSumTest (MQRankSum <−12.5), ReadPosRankSumTest (ReadPosRankSum <−8.0), StrandOddsRatio (SOR >4.0), Phred scaled quality score (QUAL <50.0); (ii) INDEL: QD <2.0, FS >200.0, ReadPosRankSum <−20.0, SOR >10.0, likelihood-based test for the inbreeding among samples (InbreedingCoeff < −0.8). The following calls were filtered by in-house program. (iii) Variants with average read depths <8; (iv) variants in the repeat regions identified by Repeat Masker or Tandem repeat finder; (v) Indel variants in homopolymer. (vi) To reduce false positives, we used a normal panel as a filter. We extracted variants using Fisher's exact test by statistically comparing the number of reference alleles and of variant alleles between 9 case samples and 130 normal samples, which were carried out by exome sequencing and variant calling using the same methods as case samples. Variants with $P \geq .05$ were excluded. ANNOVAR[27] was used to appropriately annotate the genetic variants. This included gene annotation, amino acid change annotation, SIFT score, PolyPhen2 score and dbSNP identifiers.

2.4 | Statistical analysis

Fisher's exact test and the Mann-Whitney *U* test were used to assess the significance of the associations between clinical characteristics and trastuzumab-induced cardiotoxicity. To identify rare but large effect size variants, we selected variants with MAF <0.05 in the HGVD (N_{max} = 1208), which consist of genotypes from 1208 healthy Japanese individuals,[28] after whole exome sequencing, variant annotation, and filtering. In the screening study (exome sequencing), case (9 cardiotoxicity samples)-control (1208 healthy Japanese individuals in HGVD) association study was done using Fisher's exact test in three genetic models: an allele frequency model and dominant-effect or recessive-effect model, assuming that the cardiotoxicity risk allele could contribute to adverse events in a dominant-inheritance or recessive-inheritance way. Significance levels after Bonferroni correction for multiple testing of three genetic models were $P = 7.38 \times 10^{-6}$ (0.05/[2258 × 3]) in the screening study and $P = .0019$ (0.05/[9 × 3]) in the replication study. OR and CI were calculated using the non-risk genotype as a reference. Calculation of sample size for a replication study was carried out by GDesignMini (StaGen, Tokyo, Japan).

3 | RESULTS

3.1 | Patient characteristics

To identify a genetic marker(s) determining the risk of trastuzumab-induced cardiotoxicity, we selected 243 patients treated with trastuzumab from the registered samples in NCC biobank. Table 1 shows the characteristics of these 243 patients who were treated with trastuzumab. Their median age at the beginning of trastuzumab treatment was 52 and 56 years old in case and control groups, respectively. Among the characteristics listed in Table 1, none of them showed significant differences between case and control groups.

TABLE 1 Patient demographics and clinical characteristics

Characteristic	No. of patients (%)		*P*-value
	Case (N = 19)	Control (N = 224)	
Age (y)			
Median	52	56	.20
Range	32-72	29-86	
Gender			
Female	18 (94.7)	168 (75.0)	.05
Male	1 (5.3)	56 (25.0)	
Primary cancer site			
Breast	18 (94.7)	157 (70.1)	.06
Gastric	1 (5.3)	63 (28.1)	
Others and unknown	0 (0)	4 (1.8)	
Pretreatment with anthracycline			
Yes	11 (57.9)	129 (57.6)	1.00
No	8 (42.1)	95 (42.4)	
Her-2			
Negative	0 (0)	0 (0)	1.00
Positive	19 (100)	223 (99.6)	
Unknown	0 (0)	1 (0.4)	
ER status (breast cancer)			
Negative	4 (22.2)	48 (30.6)	.30
Positive	13 (72.2)	107 (68.1)	
Unknown	1 (5.6)	2 (1.3)	
PR status (breast cancer)			
Negative	8 (44.4)	52 (33.1)	.49
Positive	10 (55.6)	104 (66.3)	
Unknown	0 (0)	1 (0.6)	

ER, estrogen receptor; PR, progesterone receptor.

3.2 | Whole exome sequencing and identification of candidate variants

We defined trastuzumab-induced cardiotoxicity as that which causes ≥10% decrease of LVEF compared to before trastuzumab treatment.[29-31] This criteria includes lower grade cardiotoxicity; however, we defined this criteria as clinically significant toxicity because lower grade trastuzumab-induced cardiotoxicity, which might often be reversible, could be a warning of life-threatening cardiotoxicity especially in old women and/or in patients with complications of heart disease. Whole exome sequencing was done using germline DNAs of 9 patients with trastuzumab-induced cardiotoxicity. We discovered a total of 239360 variants in 9 cases, with 129-fold mean depth in targeted exonic regions. Variants were filtered by using an in-house program as described in Materials and Methods, and a total of 2258 genetic variants were selected. We conducted a case-control association study between 9 cases (with trastuzumab-induced cardiotoxicity) and 1208 (maximum) general Japanese population controls registered in HGVD to find the genetic variants (MAF <0.05) associated with risk of trastuzumab-induced cardiotoxicity in the screening study. Although no variants reached a significance level ($P = 7.38 \times 10^{-6}$; see Materials and Methods), we observed 10 variants showing $P_{min} < .001$ as shown in Table 2. The top variant which showed the lowest *P*-value in the screening study was rs139503277 (non-synonymous variant) in PHD Finger Protein 3 (*PHF3*) (P_{min} = .00012, OR = 51.23 with 95% CI of 11.34-231.37; Table 2).

3.3 | Replication and combined study using additional patients

To further validate the results of the screening study, we carried out a replication study of 10 variants showing $P_{min} < .001$ in the screening study using 234 independent patients, including 10 cases and 224 controls (showing no sign of trastuzumab-induced cardiotoxicity), because the power calculation using screening data indicated that 10 cases and ≥188 controls were needed in a replication study

TABLE 2 Summary of results for the screening study of 10 variants that are possibly associated with trastuzumab-induced cardiotoxicity ($P < .001$ in the screening study)

Chr.	SNP ID	Position	Gene	Allele (1/2)[a]	Risk allele	Case				Control				P-value			Odds ratio (95% CI)		
						Genotype			RAF	Genotype			RAF	Allelic	Dominant	Recessive	Allelic	Dominant	Recessive
						11	12	22		11	12	22							
6	rs139503277	64,394,450	*PHF3*	G/A	A	6	3	0	0.167	1127	11	0	0.005	.00014	.00012	1.00	41.2 (10.4-162.7)	51.2 (11.3-231.4)	NA
2	rs146213213	197,637,855	*GTF3C3*	G/A	A	6	3	0	0.167	1123	17	0	0.007	.00042	.00036	1.00	26.6 (7.1-100.5)	33.0 (7.6-143.1)	NA
17	rs78272919	39,673,366	*KRT15*	C/G	G	5	4	0	0.222	1077	47	0	0.021	.00055	.00039	1.00	13.4 (4.2-42.2)	18.3 (4.8-70.5)	NA
19	rs140387622	54,376,859	*MYADM*	G/A	A	5	3	1	0.278	1072	76	4	0.036	.00043	.0024	.038	10.2 (3.5-29.2)	10.7 (2.8-40.7)	35.9 (3.6-357.5)
10	rs150273659	81,318,681	*SFTPA2*	G/A	A	6	3	0	0.167	1086	19	1	0.009	.00080	.00060	1.00	20.9 (5.6-77.5)	27.2 (6.3-116.3)	0
22	rs5762940	29,383,081	*ZNRF3*	A/G	G	6	2	1	0.222	1092	48	1	0.022	.00065	.0060	.016	12.8 (4.1-40.1)	11.1 (2.7-45.9)	142.5 (8.2-2482.6)
1	rs149581993	204,216,592	*PLEKHA6*	C/T	T	7	2	0	0.111	797	2	0	0.001	.00069	.00065	1.00	99.8 (13.2-752.6)	113.9 (14.0-926.5)	NA
6	rs139944387	64,430,690	*EYS*	T/C	C	6	3	0	0.167	1118	23	0	0.010	.00094	.00079	1.00	19.6 (5.3-72.5)	24.3 (5.7-103.2)	NA
5	rs201763080	172,518,046	*CREBRF*	C/T	T	7	2	0	0.111	1098	4	0	0.002	.00091	.00086	1.00	68.8 (11.7-402.5)	78.4 (12.3-500.3)	NA
6	rs56378532	110,036,274	*FIG 4*	T/C	C	5	4	0	0.222	1082	59	2	0.028	.0015	.00094	1.00	10.1 (3.2-31.5)	14.2 (3.7-54.2)	0

Chr., chromosome; CI, confidence interval; NA, not available; RAF, risk allele frequency; SNP, single nucleotide polymorphism.

[a]Major allele in control is defined as allele 1.

to achieve a statistical power of at least 0.8 for the top 10 SNV of the screening study. We set significance levels for three genetic models after the Bonferroni correction at *P*-values of .0019 (0.05/[9 × 3]) in the replication study because two variants were highly linked ($r^2 = .85$) to the other variant. We could not identify any variants which showed significance levels of association in the replication study after the Bonferroni correction. This might be because of the insufficient sample size in the replication study; however, we observed that three variants had an effect in the same direction as in the screening study (rs78272919 in exon 2 of Keratin 15 (*KRT15*), rs5762940 in exon 2 of zinc and ring finger 3 (*ZNRF3*), and rs139944387 in exon 44 of Eyes shut homologs (*EYS*); Table S1). The number of samples used for the screening and the replication studies was not large enough; however, these variants might be candidates to be associated with trastuzumab-induced cardiotoxicity. A combined result of the screening and the replication studies showed stronger association (smaller *P*-value) of a locus on chromosome 6q12 with trastuzumab-induced cardiotoxicity compared with the result in the screening study (rs139944387 in *EYS*, combined-$P_{min} = .00056$, OR = 13.73 with 95% CI of 4.27-44.21, Table 3).

4 | DISCUSSION

As no clinically applicable biomarker for trastuzumab-induced cardiotoxicity has been developed, in the present study, we conducted whole exome sequencing using germline DNA samples from 9 patients with trastuzumab-induced cardiotoxicity and identified a locus possibly associated with cardiotoxicity (Table 3). In our study, none of the top 10 SNV in the screening phase achieved $P < .05$ in the replication study. Larger sample size might be needed to achieve $P < .05$ in the replication study because, in this study, the sample size calculated using screening data (10 cases and ≥188 controls) might not be sufficient as a result of the overestimation of odds ratio in the screening phase. The associated variant, rs139944387, was located in exon 44 of *EYS*, which encodes the eyes shut homolog (also known as Epidermal Growth Factor-Like Protein 10 or 11). This variant is a synonymous variant, and is classified as having "uncertain clinical significance" in the ClinVar database. *EYS* spans over 2 Mb of genomic DNA, consists of 44 exons, and is expressed in normal tissues including fat, colon, heart, and retina according to the gene expression database for normal tissues.[32-34] Mutations in *EYS* are known to be a common cause of autosomal recessive retinitis pigmentosa.[33,35] The EYS protein consists of a signal peptide followed by EGF-like domains, putative coiled-coil domain and laminin G-like domains which are interposed by EGF-like repeats.[36,37] Moreover, the signal peptide and the cleavage site in the N-terminal region are predicted in EYS, suggesting that EYS is a secreted protein.[37] HER2, which is the target of trastuzumab, is one of the members of the EGFR family, and 11 ligands for EGFR including EGF, transforming growth factor alpha (TGFA), and amphiregulin (AREG) have been identified.[38-41] After the above ligand binds to the extracellular domain of the EGFR(s), the receptor forms functionally active

TABLE 3 Association results of rs139944387

Chr.	SNP ID	Position	Gene	Allele (1/2)[a]	Stage	Risk allele	Case Genotype 11	12	22	RAF	Control Genotype 11	12	22	RAF	P-value Allelic	Dominant	Recessive	Odds ratio (95% CI) Allelic	Dominant	Recessive
6	rs139944387	64,430,690	*EYS*	T/C	Screening	C	6	3	0	0.167	1118	23	0	0.010	.00094	.00079	1.00	19.6 (5.3-72.5)	24.3 (5.7-103.2)	NA
					Replication		9	1	0	0.050	221	3	0	0.007	.16	.16	1.00	7.8 (0.8-78.6)	8.2 (0.8-86.6)	NA
					Combined		15	4	0	0.105	1339	26	0	0.010	.00064	.00056	1.00	12.2 (4.0-37.0)	13.7 (4.3-44.2)	NA

Chr., chromosome; CI, confidence interval; NA, not available; RAF, risk allele frequency; SNP, single nucleotide polymorphism.
[a]Major allele in control is defined as allele 1.

dimers (homodimer, eg, EGFR-EGFR or heterodimer, eg, EGFR-HER2).[42,43] It is reported that HER2 is known to be the potent heterodimerization partner for all EGFR to elicit signaling pathways[44,45] and that HER2 signal is important in cardiac homeostasis.[46] Although we do not have evidence that EYS could be a novel ligand for EGFR including HER2, these lines of evidence suggest that EYS might affect the efficiency of HER2 signal transduction in cardiac myocytes, and interindividual difference of EYS function caused by the genetic variant(s) might affect the incidence of trastuzumab-induced cardiotoxicity. However, further functional analysis will be needed to clarify the biological mechanisms that could have effects on trastuzumab-induced cardiotoxicity.

In the replication study, we observed that rs5762940 in exon 2 of *ZNRF3* had an effect in the same direction as in the screening study. ZNRF3 negatively regulates Wnt/β catenin signaling through promoting turnover of frizzled and LRP6.[47,48] Moreover, Wnt/β catenin signaling is suggested to suppress ERBB signaling through down-regulation of neuregulin, which is one of the ligand for ERBB.[49,50] Hence, inactivation or reduced expression of ZNRF3 might induce ERBB signal inhibition in cardiac myocytes through activation of Wnt/β catenin signaling, and might cause trastuzumab-induced cardiotoxicity, although further work will be necessary to elucidate the mechanisms that could have effects on trastuzumab-induced cardiotoxicity.

rs1058808 (P1170A) and rs1136201 (I655V) in *ERBB2* have been suggested to be candidate variants that confer risk of developing trastuzumab-induced cardiotoxicity mainly in Caucasians; however, the association results are still controversial.[24,25,51,52] In our study, the two SNP were not associated with trastuzumab-induced cardiotoxicity (rs1058808; combined-P_{min} = .42, OR = 0.75 with 95% CI of 0.39-1.43, rs1136201; combined-P_{min} = .35, OR = 0.50 with 95% CI of 0.15-1.62). Allelic frequencies of the risk alleles of the two SNP are 0.33 (rs1058808) and 0.25 (rs1136201) in Caucasians; however, 0.44 and 0.13 in Japanese, respectively. Interethnic difference of allele frequencies of the two SNP might also be one of the causes of replication failure in our study.

In conclusion, our sequencing analysis using 243 Japanese patients treated with trastuzumab identified a novel variant in the *EYS* gene associated with trastuzumab-induced cardiotoxicity. This finding provides new insights into personalized trastuzumab therapy for patients with HER2 positive cancer. However, a large-scale replication study and further functional analysis are required to validate our results and to elucidate the biological mechanisms that have effects on this variant on the risk of trastuzumab-induced cardiotoxicity.

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CONFLICTS OF INTEREST

Authors declare no conflicts of interest for this article.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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