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ORIGINAL ARTICLE: RESEARCH

Discovery of donor genotype associated with long-term survival of patients with hematopoietic stem cell transplantation in refractory acute myeloid leukemia

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ABSTRACT

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) has been the only treatment option for acute myeloid leukemia (AML) refractory to induction chemotherapy, with only 10–20% of patients achieving long-term survival. Certain donor genotypes may confer leukemia-clearing effects after allo-HSCT. We performed whole-exome sequencing of five pairs of the germ lines in AML patients who achieved long-term remission after allo-HSCT and in their donors, and found two significant variants: *EGFR* c.2982C > T and *CDH11* c.945G > A. To validate the protective effects of these leukemia-clearing genotypes (LCGs), AML patients who received allo-HSCT in a complete-remission status were also analyzed. Twenty-two of 96 donors (22.9%) had LCGs in their genomes, and overall survival was significantly longer in patients who received allo-HSCT from donors with germ-line LCGs (hazard ratio=0.47, 95% confidence interval=0.24–0.94, $p = .033$). These findings indicate that donor germ-line LCGs have phenotypically leukemia-clearing effects and are biomarkers for predicting clinical outcomes in allogeneic transplantation in AML patients.

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Introduction

It is estimated that there are about 60,000 new cases of leukemia annually in the US, among which acute myeloid leukemia (AML) has the highest incidence and is the most common cause of death [1]. The treatment strategy for AML is well defined according to risk stratification, and long-term remission is achieved only with chemotherapy in favorable risk groups. However, certain AML patients are refractory to induction or salvage chemotherapy [2], and allogeneic hematopoietic stem cell transplantation (allo-HSCT) is the only option for curative treatment in chemorefractory AML [3]. However, allo-HSCT results in long-term survival in less than 20% of patients with refractory AML [4,5], and so the factors associated with a favorable clinical outcome of allo-HSCT in refractory AML need to be identified.

Various factors reportedly influence the outcome of allo-HSCT in patients with refractory AML [4,6,7]. Duval et al. performed the largest study, which showed that fewer than two chemotherapy regimens before allo-HSCT, long duration of the first complete remission (CR), dose of infused CD34 cells, absence of blasts in peripheral blood, and a human leukocyte antigen (HLA)-identical donor are associated with longer overall survival (OS) [4]. However, whether a specific donor genotype other than the HLA type is related to the clinical outcome has not been reported previously.

In allo-HSCT for chemorefractory AML, transplant conditioning such as high-dose chemotherapy or radiation might not contribute as much as the graft-versus-leukemia (GVL) effect for controlling leukemia, since leukemic cells exhibit chemoresistance. The GVL effect has traditionally been known to be influenced by HLA. Donor versus recipient immune reactions are

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Supplemental data for this article can be accessed here.

also known to be influenced by polymorphisms in certain genes coding for antigen-presenting molecules, antigen receptors, immune mediators, or cellular proliferation molecules [8]. Likewise, we cannot exclude the possibility that a specific donor genotype other than HLA would be significantly associated with the clinical outcome of a patient.

In the case of long-term survival after sibling HSCT, any germ-line genomic difference between the donor and patient could be the key factor contributing to leukemia development and chemorefractoriness and, in contrast, a leukemia-clearing effect. Knowledge of which genomic alteration is associated with this possibility of leukemia clearing would be very informative when choosing the donor from among candidates. Moreover, this genomic factor might provide important clues for understanding anticancer treatment in general.

In the current study, we hypothesized that certain donor genotypes confer leukemia-clearing effects after allo-HSCT in long-term survivors of refractory AML. To discover the shared specific donor genotype, we analyzed the genomic differences between sibling donors and patients in a specific group. We selected patients in a chemorefractory status who achieved long-term survival with allo-HSCT.

Patients and methods

Patient cohort

We retrospectively reviewed all cases of AML that received allo-HSCT at Seoul National University Hospital (SNUH) from 1985 to 2015. In order to discover donor-specific protective factors in these cases, we focused on chemotherapy-refractory AML cases that achieved long-term survival after allo-HSCT (the discovery cohort). The primary objective of the discovery cohort was to enrich the specific genotype of the donor germ line related to the long-term survival of AML patients after allo-HSCT. To validate these protective effects, AML patients who received allo-HSCT in a CR status were also enrolled (the HSCT-in-CR cohort). We collected information on the age at allo-HSCT, sex, cytogenetic risk stratification, disease status at allo-HSCT, previous hematologic malignancy including myelodysplatic syndrome, donor type (related or unrelated), and OS after allo-HSCT in the SNUH validation cohort.

The primary objective of using the SNUH validation cohort was to validate the protective effects of specific donor genotypes analyzed from the discovery cohort in an independent patient group. Only donors and patients who provided informed consent for the use of their genomic DNA were enrolled in the current study. This study was conducted according to the Declaration of Helsinki and was approved by the institutional review board (IRB) of SNUH (IRB No. H-1507-068-688).

Whole-exome sequencing and analysis

Exome sequencing of germ-line DNA was performed using the Ion AmpliSeq^{TM} Exome Panel to screen the coding sequence region of the entire genome. This panel included the exome of 19,072 genes, and the size of the total targeted region was 57.7 Mb. The panel contained 293,903 primer pairs that were multiplexed into 12 pools to avoid primer-dimer formation and interference during the polymerase chain reaction (PCR). The range of amplicons amplified by these oligo primer pairs ranged from 125 to 275 bp, and the rate of on-target coverage for this panel was 95.69%. PCR assays were performed directly to amplify 100 ng of genomic DNA samples to collect the target regions using the oligo primer pairs of the panel. The reaction conditions were as follows: 99 °C for 2 min, followed by 10 cycles of 99 °C for 15 sec, 60 °C for 16 min, and 10 °C for 1 min. After amplification, a library was constructed using the Ion AmpliSeq 2.0 library kit as described in the manufacturer's instructions (Life Technologies, Carlsbad, CA). Libraries were quantified using the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA) and then diluted to ~10 pM. Subsequently, 50 μL of the barcoded libraries were combined in sets of two barcodes, and the combined libraries were sequenced using the Ion Proton platform following the manufacturer's instructions (Life Technologies). A mean depth of sequencing that ranged from 100× to 140× was obtained, which was sufficient to interrogate the target regions for mutations. Raw reads were mapped to the human reference genome (GRCh37) using the Torrent Mapping Alignment Program in the Torrent Suite software (Thermo Fisher). Variants were then identified with the Genome Analysis Toolkit (version 2.8) software [9] using HaplotypeCaller, which is capable of calling single-nucleotide polymorphisms (SNPs) and insertion/deletion polymorphisms simultaneously via local *de novo* assembly of haplotypes in a given region.

The following criteria were applied to select variants (Figure 1): (i) all sequence variants found in donor samples but not in recipient samples were selected; (ii) coding DNA sequence (CDS) variants were selected based on exon information in the UCSC Genomes Browser [10]; (iii) variants in chromosome Y were excluded; (iv) we calculated allele carrier

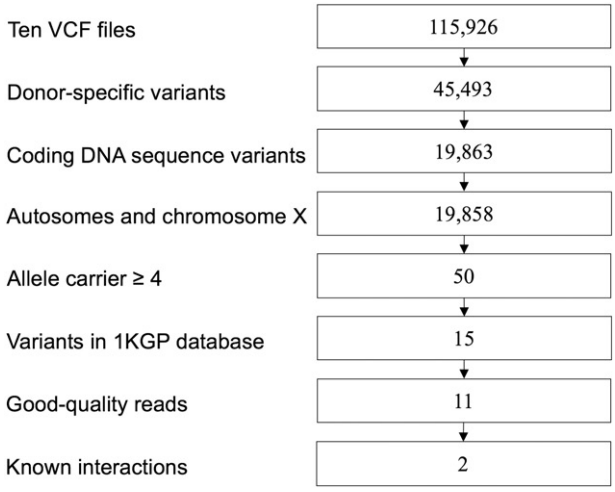

Figure 1. Flow diagram of the number of variants in each filtering step. 1KGP: the 1000 Genomes Project; VCF: variant call format.

frequencies and selected variants if they were found in more than four (80%) of the five included donors; (v) variants reported in the 1000 Genomes Project [11] were selected since they are less likely to be false positives; (vi) for selected variants, we manually inspected the alignment statuses in BAM and determined whether a variant was a false positive [12]; and finally (vii) we found known interactions between any pairs of genes by searching the PubMed and Pathway databases.

SNP genotyping

The two SNPs *EGFR* c.2983C > T (rs2293347) and *CDH11* c.945G > A (rs28216) (Nos. C__15970737_20 and C___3299779_10 in Assay-on-Demand, Thermo Fisher Scientific, South San Francisco, CA) were genotyped in the HSCT-in-CR cohort using the TaqMan assay [13] performed with the following steps: (i) preparation of approximately 20 ng of purified genomic DNA; (ii) preparation of genotyping mixture consisting of 2X genotyping master mix, 20X SNP genotyping assay, DNAse-free water, and template DNA; and (iii) PCR comprising 40 cycles of denaturation and annealing/extension steps. When the PCR was completed, genotypes of the DNA samples were analyzed on a sequence detection system (ABI prism 7900HT, Applied Biosystems, Foster City, CA). Quality control of the genotyping was performed by duplicate checking of 10% of the samples, which revealed a rate of concordance in duplicates of >99.5%.

Statistical analysis

OS was measured in the discovery and HSCT-in-CR cohorts from the first day of allo-HSCT until death or the last follow-up date, if censored. Survival analyses of OS were carried out according to the Kaplan-Meier method with log-rank testing to assess intergroup differences. A Cox proportional-hazards regression model was used to calculate hazard ratio (HR) and 95% confidence interval (CI) values for effects of the donor-specific genotype on the patient's OS. The chi-square test was used to determine the significance of the associations of clinicopathologic parameters according to genomic characteristics. All reported p values were two-sided, and considered significant if $p < .05$. Statistical analyses and data presentations were performed using STATA software (version 12, StataCorp, College Station, TX).

Results

Patient characteristics in the discovery cohort

Overall, 346 AML patients had undergone allo-HSCT, and only 16 of 56 refractory cases achieved long-term survival thereafter (Supplementary Figure S1). Among them, five sibling pairs were enrolled and agreed to contribute their genomic DNA in the training cohort, and were finally analyzed (discovery cohort; Table 1). All of the patients in the discovery cohort were refractory to induction chemotherapy and salvage chemotherapy, and had more than 34% of leukemic blasts in their bone marrow at allo-HSCT.

Whole-exome sequencing findings

Whole-exome sequencing was applied to five donor–-recipient matched pairs. The workflow of our study is presented in Figure 1. After sequencing, 115,926 variants were identified in 10 samples. We first selected 45,493 variants that were reported in the donor group

Table 1. Summary of the donors and recipients included in the discovery cohort.

Donors				Recipients					
No.	Gender	Age, years	Relation	Sex	Age, years	Diagnosis	Risk level	Bone marrow blasts at HSCT	OS after HSCT
1	Female	25	Sibling	Male	27	AML	Standard	Normoblasts, 34.0%	124 months, survived
2	Male	41	Sibling	Male	45	AML	Standard	Myeloblasts, 84.0%	140 months, survived
3	Male	23	Sibling	Male	26	AML	Standard	Blasts, 51.4%	85 months, survived
4	Male	55	Sibling	Female	48	AML	Standard, MDS history+	Normoblasts, 35.4%	71 months, survived
5	Male	56	Sibling	Male	52	AML	High (FLT3-ITD+), MDS history+	Myelocytes, 70.8%	31 months, died

AML: acute myeloid leukemia; FLT3-ITD: FLT3 internal tandem duplication; HSCT: hematopoietic stem cell transplantation; MDS: myelodysplastic syndrome; OS: overall survival.

but not the recipient group, and then 19,863 variants in CDS regions were selected. We excluded variants in chromosome Y, resulting in 19,858 variants remaining. There were 50 variants in which a variant was shared by more than 4 of the 5 donors. Sixteen variants that were found in the 1000 Genome Project (1KGP) were selected based on the hypothesis that a variant reported in a public database could be highly curated. Misalignment or incorrect enzymatic reactions resulted in the exclusion of four false-positive variants by inspecting the alignment statuses in BAM files. This resulted in the following 11 variants within 10 genes remaining as the best candidates for being involved in leukemia-clearing events: *CCSER1*, *CDH11*, *CFAP65*, *EGFR*, *GRIN2B*, *MICALL1*, *MOV10*, *TMEM221*, *TNNT3*, *TNRC18*, and *ZNF177*.

When testing for canonical pathways in our data set, the PANTHER Pathway 3.4 [14] included some of our more interesting candidates, which was an interaction between *EGFR* and *CDH11*: '*EGFR* changes the state of *CDH11*'. Notably, all of the donors were heterozygous for the *EGFR* c.2982C > T variant, and the minor allele frequency (MAF) of this variant is higher in East Asian (MAF = 0.2470) and South Asians (0.2301) Asian than in Americans (0.1542), Europeans (0.1113), and Africans (0.0129) in 1KGP. Therefore, *EGFR* c.2982C > T and *CDH11* c.945G > A variants were finally filtered as donor-specific genotypes and so are called leukemia-clearing genotypes (LCGs) hereafter (Table 2).

Biologic validation of donor LCGs of allo-HSCT in a CR status

To validate the biologic significance of LCGs in allo-HSCT, we constructed an independent validation cohort consisting of AML patients who underwent allo-HSCT in a CR status (Supplementary Figure S1). In the HSCT-in-CR cohort, 22 of 96 donor–patient pairs (22.9%) had both LCGs in their germ-line DNA (Table 3 and Supplementary Figure S2). The general patient characteristics did not differ significantly according to the donor LCG status. OS in the HSCT-in-CR cohort did not differ significantly according to the donor genotype of either *EGFR* c.2982C > T ($EGFR^{HET(heterozygous)}$ or $EGFR^{HOM(homozygous)}$) or *CDH11* c.945G > A ($CDH11^{HET}$ or $CDH11^{HOM}$), although each genotype seemed to be associated with longer survival (Supplementary Figure S3). A particularly interesting observation was that OS was significantly longer in patients who received HSCT from donors who had both LCGs (*EGFR* c.2982C > T [$EGFR^{HET}$ or $EGFR^{HOM}$] and *CDH11* c.945G > A [$CDH11^{HET}$ or $CDH11^{HOM}$]) than in those whose donor did not have both LCGs (HR = 0.47, 95% CI = 0.24–0.94, $p = .033$; Figure 2). A univariate Cox regression analysis of the HSCT-in-CR cohort revealed that the patient's age, cytogenetic risk, and the donor's LCG status were significantly associated with the prognosis. In multivariate analysis, the donor's LCG status was related to a favorable prognosis, with an adjusted HR of 0.55 (95% CI = 0.27–1.12; Supplementary Table S1).

Discussion

This study has shown that two LCGs (*EGFR* c.2982C > T and *CDH11* c.945G > A) were shared in sibling donors who rescued chemorefractory AML patients. In another donor–patient cohort in which allo-HSCT was performed in a CR status, 22% of donors had both LCGs (or at least the haplotype for each LCG), and patients who received allo-HSCT from LCGs-harboring donors had a significantly longer OS. Here, we report the specific donor genotypes that are predictive of clinical outcomes of AML in allo-HSCT.

EGFR is a well-known oncogene whose mutation is known to be directly related to many solid cancers, including non-small-cell lung cancer (NSCLC) [15,16]. Although a variant found in our study is a synonymous one (D994D), the *EGFR* c.2982C > T variant is reportedly associated with the response to gefitinib (an EGFR inhibitor) in *EGFR*-mutated NSCLC [17], suggesting the functionality of this genetic change. On the other hand, *CDH11* encodes a type II classical cadherin that mediates calcium-dependent cell–cell adhesion [18]. *CDH11* acts as an important biomarker in

Table 2. Leukemia-clearing genotypes of the donor germ-line DNA.

Gene	Chr	Position	rsID	Ref	Alt	Type	HGVS.c	HGVS.p	FATHMM	CADD	MAF
EGFR	7	55268916	rs2293347	C	T	Synonymous	c.2982C > T	p.Asp994Asp	0.91297	11.96	0.1418
CDH11	16	65022114	rs28216	C	T	Synonymous	c.945G > A	p.Ser315Ser	0.91039	19.86	0.2236

Alt: alternative allele; CADD: Combined Annotation Dependent Depletion; Chr: chromosome no.; FATHMM: Functional Analysis Through Hidden Markov Models; HGVS: Human Genome Variation Society sequence variation nomenclature; MAF: minor allele frequency [from phase 3 of the 1000 Genomes Project ($N = 2504$)]; Ref: reference allele.

many types of cancer [19–21], which implies that this gene is important in cancer. The variant found in *CDH11* in our study c.945C > T is annotated in the dbSNP [22], and its biologic function has not been reported yet. However, algorithms for predicting biologic significance *in silico* have consistently called these two variants pathogenic: FATHMM (Functional Analysis Through Hidden Markov Models, version 2.3) [23] and CADD (Combined Annotation Dependent Depletion, version 1.3) [24]; this suggests the biologic relevance of our findings. Since it would be very difficult to perform functional validation of such germ-line synonymous variants, we believe the biologic impact of LCGs should be validated in an independent large allo-HSCT cohort.

The biologic validation of LCGs was conducted in the HSCT-in-CR cohort. Since we had already analyzed the shared genotypes in donors paired with chemorefractory AML patients in allo-HSCT at SNUH, we constructed an independent cohort in a considerably different clinical setting for the biologic validation of LCGs. If transplantation from the LCG carrier would relate to a longer OS, this effect might also occur in a general allo-HSCT case performed in a CR status; that is, in our HSCT-in-CR cohort. Interactive analysis of the HSCT-in-CR cohort produced two important results: (i) the frequency of LCGs in the general population and (ii) the survival benefit of LCGs transplantation. Since the frequency of LCGs would be related to the long-term survival rate of AML by allo-HSCT, we

Table 3. Patient characteristics in the HSCT-in-CR cohort.

	Total $N = 96$	*EGFR*^{WT} *CDH11*^{WT} $N = 21$	*EGFR*$^{HET/HOM}$ *CDH11*^{WT} $N = 30$	*EGFR*^{WT} *CDH11*$^{HET/HOM}$ $N = 23$	*EGFR*$^{HET/HOM}$ *CDH11*$^{HET/HOM}$ $N = 22$	*p*
Age at HSCT						
Years	47 (18–67)	46 (19–63)	48 (18–67)	49 (20–62)	39 (20–64)	.584
Gender						
Male	57 (59.4)	12 (57.1)	19 (63.3)	16 (70.0)	10 (45.5)	.403
Female	39 (40.6)	9 (42.9)	11 (36.7)	7 (30.4)	12 (54.5)	
Cytogenetic risk stratification						
Favorable	24 (25.0)	3 (14.3)	8 (26.7)	6 (26.1)	7 (31.8)	.432
Standard	34 (35.4)	12 (57.1)	8 (26.7)	8 (34.8)	6 (27.3)	
Poor	38 (39.6)	6 (28.6)	14 (46.6)	9 (39.1)	9 (40.9)	
Disease status						
CR1	47 (49.0)	10 (47.6)	13 (43.3)	13 (56.5)	11 (50.0)	.566
Delayed CR1	5 (5.2)	11 (52.4)	14 (46.7)	8 (34.8)	11 (50.0)	
CR2	44 (45.8)	0 (0)	3 (10.0)	2 (8.7)	0 (0)	
Previous MDS						
No	71 (74.0)	13 (61.9)	23 (76.7)	15 (65.2)	20 (90.9)	.103
Yes	25 (26.0)	8 (38.1)	7 (23.3)	8 (34.8)	2 (9.1)	
Donor type						
Related	51 (53.1)	9 (42.9)	17 (56.7)	11 (47.8)	14 (63.6)	.520
Unrelated	45 (46.9)	12 (57.1)	13 (43.3)	12 (52.2)	8 (36.4)	
GVHD after HSCT						
No	62 (64.6)	15 (71.4)	21 (70.0)	11 (47.8)	15 (68.2)	.313
Yes	34 (35.4)	6 (28.6)	9 (30.0)	12 (52.2)	7 (31.8)	
HLA matched						
Matched	88 (91.7)	19 (90.5)	27 (90.0)	22 (95.7)	20 (90.9)	.886
Unmatched	8 (8.3)	2 (9.5)	3 (10.0)	1 (4.3)	2 (9.1)	
Conditional regimen						
BuFluATG	53 (55.2)	12 (57.1)	16 (53.3)	13 (56.5)	12 (54.6)	.725
BuCy	27 (28.1)	8 (38.1)	8 (26.7)	5 (21.7)	6 (27.3)	
OS after HSCT	16 (16.7)	1 (4.8)	6 (20.0)	5 (21.7)	4 (18.2)	
Others						
OS after HSCT						
Months	14.3 [9.4–22.3]	17.4 [6.7–N/R]	10.1 [4.0–50.6]	12.1 [6.4–20.7]	N/R [8.4–N/R]	.099

Data are *N* (%), median (range), or median [95% confidence interval] values.
BuCy, busulfan, cyclophosphamide; BuFluATG, busulfan, fludarabine, antithymocyte globulin; CR: complete remission; CR1/2: first/second complete remission; GVHD: graft-versus-host disease; HET: heterozygous; HLA: human leukocyte antigen; HOM: homozygous; N/R: not reached; WT: wild type.

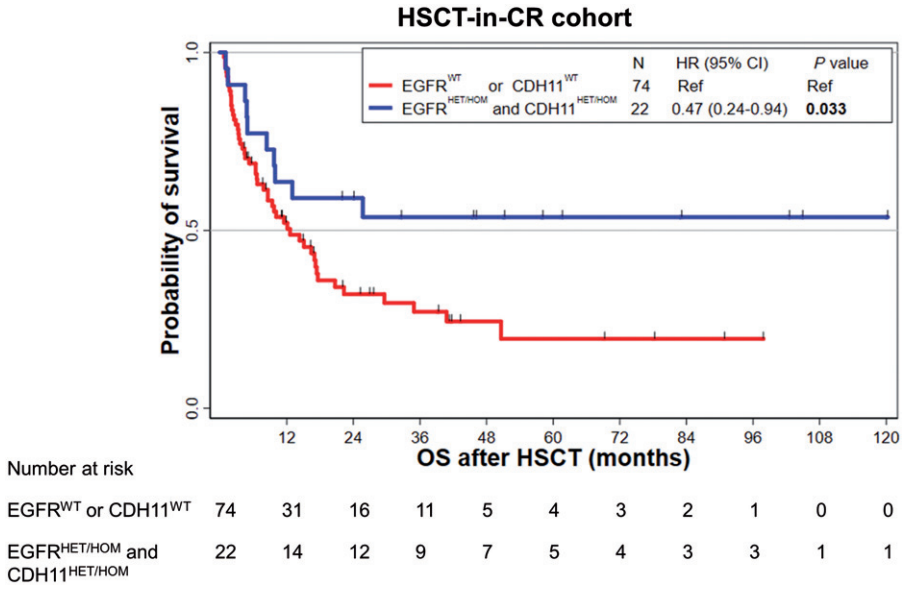

Figure 2. Survival analysis in the HSCT-in-CR cohort according to donor leukemia-clearing genotypes. Kaplan–Meier curves of overall survival (OS) according to whether (blue line) or not (red line) the corresponding donor carried both germ-line variants of *EGFR* c.2982C > T and *CDH11* c.945G > A. CI: confidence interval; CR: complete remission HET: heterozygous; HOM: homozygous; HR: hazard ratio; HSCT: hematopoietic stem cell transplantation; Ref: reference; WT: wild type.

hypothesized that the estimated LCGs frequency would be within the range of 10–30% in a general population. Among our 96 donor and patients pairs in the HSCT-in-CR cohort, 22.9% of the donors harbored both LCGs, which was consistent with our preliminary hypothesis. Even more interestingly, patients who received transplantation with both LCGs showed a significantly prolonged survival. However, we could not obtain straightforward evidence for the biologic mechanism underlying how *EGFR* c.2982C > T and *CDH11* c.945G > A would produce an antitumor environment.

There are some weak points of our analysis. Due the extreme difficulty of achieving a pure germ-line DNA without tumor contamination for both donors and patients who were refractory to chemotherapy, only five donor–patient pairs were analyzed for the extraction of LCGs, and we cannot exclude the possibility of there being LCG variants other than *EGFR* c.2982C > T and *CDH11* c.945G > A. In addition, the biologic validation of LCGs in the HSCT-in-CR cohort was conducted in AML patients who received allo-HSCT in CR rather than in an independent cohort of chemorefractory AML patients. However, we observed the same leukemia-clearing effect in this validation cohort and believe that this validation result does not weaken our conclusion, since the leukemia-clearing effect by LCG carriers would be more diluted in a CR cohort than in a chemorefractory cohort. However, we admit that a large patient cohort needs to be analyzed to confirm our observations and utilize our findings in clinical applications. Lastly, considering that both *EGFR* and *CDH11* variants are synonymous, the bridging mechanism underlying how these genotypes have the ability to clear leukemia remains to be elucidated.

In conclusion, the germ-line variants of *EGFR* c.2982C > T and *CDH11* c.945G > A, or LCGs, have phenotypically leukemia-clearing effects when performing transplantation in AML patients. Future prospective studies should evaluate if donors with germ-line LCGs are related to a favorable clinical outcome of allo-HSCT.

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