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## **Killer Immunoglobulin-Like Receptor Allele Determination Using Next-Generation Sequencing Technology**

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# Killer Immunoglobulin-Like Receptor Allele Determination Using Next-Generation Sequencing Technology

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The impact of natural killer (NK) cell alloreactivity on hematopoietic stem cell transplantation (HSCT) outcome is still debated due to the complexity of graft parameters, HLA class I environment, the nature of killer immunoglobulin-like receptor (KIR)/KIR ligand genetic combinations studied, and KIR<sup>+</sup> NK cell repertoire size. KIR genes are known to be polymorphic in terms of gene content, copy number variation, and number of alleles. These allelic polymorphisms may impact both the phenotype and function of KIR<sup>+</sup> NK cells. We, therefore, speculate that polymorphisms may alter donor KIR<sup>+</sup> NK cell phenotype/function thus modulating post-HSCT KIR<sup>+</sup> NK cell alloreactivity. To investigate KIR allele polymorphisms of all KIR genes, we developed a next-generation sequencing (NGS) technology on a MiSeq platform. To ensure the reliability and specificity of our method, genomic DNA from well-characterized cell lines were used; high-resolution KIR typing results obtained were then compared to those previously reported. Two different bioinformatic pipelines were used allowing the attribution of sequencing reads to specific KIR genes and the assignment of KIR alleles for each KIR gene. Our results demonstrated successful long-range KIR gene amplifications of all reference samples using intergenic KIR primers. The alignment of reads to the human genome reference (hg19) using BiRD pipeline or visualization of data using Profiler software demonstrated that all KIR genes were completely sequenced with a sufficient read depth (mean 317x for all loci) and a high percentage of mapping (mean 93% for all loci). Comparison of high-resolution KIR typing obtained to those published data using exome capture resulted in a reported concordance rate of 95% for centromeric and telomeric KIR genes. Overall, our results suggest that NGS can be used to investigate the broad KIR allelic polymorphism. Hence, these data improve our knowledge, not only on KIR<sup>+</sup> NK cell alloreactivity in HSCT but also on the role of KIR<sup>+</sup> NK cell populations in control of viral infections and diseases.

**Keywords:** high-resolution killer immunoglobulin-like receptor typing, allele polymorphism, next-generation sequencing, International Histocompatibility Workshop DNA samples

## INTRODUCTION

Hematopoietic stem cell transplantation (HSCT) provides a curative therapy for many patients with hematological malignancies (1). Donors for HSCT are currently selected based on the level of matching for HLA-A, -B, -C, -DRB1, and -DQB1 loci. Siblings, 10/10 HLA matched, remain the gold standard. However, substantial risks of morbidity and mortality caused by disease relapse (2), graft-vs-host-disease (GvHD) (3), and infection (4) are still prevalent after related, or unrelated HSCT. Natural killer (NK) cells are the first post-HSCT cells, reconstituting antiviral and antitumoral activity (5). NK cells are able to recognize the missing-self *via* killer immunoglobulin-like receptors (KIRs) (6). Ruggeri et al. (7) were first to report the beneficial effect of KIR ligand mismatched donor NK cell alloreactivity after T cell-depleted HLA haplo-identical HSCT resulting in less relapse, less GvHD, and better overall survival in patients with acute myeloid leukemia. The impact of KIR<sup>+</sup> NK cell alloreactivity on HSCT outcome is still controversial due to the heterogeneity of graft parameters, HLA class I environment, nature of KIR/KIR ligand genetic combinations studied, and KIR<sup>+</sup> NK cell repertoire size (8–12).

As HLA class I genes, KIR genes are highly polymorphic (13). In humans, 16 KIR genes have been described including eight inhibitory genes (2DL1/L2/L3/L4/L5, 3DL1/L2/L3), 6 activating genes (2DS1/S2/S3/S4/S5, 3DS1), 2 two pseudogenes (2DP1, 3DP1). These genes are located within the leukocyte receptor cluster found on chromosome 19q13.4, spanning a region of 150 kb. Within a population, the genotypic diversity of KIR genes occurs at different levels. First, the number and nature of KIR genes vary between individuals defining different KIR haplotypes. KIR haplotypes are classified into group A and group B (14). The group A haplotype is defined by a fixed set of nine KIR genes: four framework KIR genes (3DL3, 3DP1, 3DL2, and 2DL4) that form the centromeric and telomeric part of KIR locus, three inhibitory KIR (2DL1, 2DL3, and 3DL1), a pseudogene (2DP1), and a single activating KIR gene (2DS4). The group B haplotype is defined as having a variable number of KIR genes (7–14) including the four framework KIR genes and specific KIR genes (2DS2, 2DL2, 2DL5, 2DS3, and 2DS1). Second, a variable number of copies [copy number variation (CNV)] of the gene generated by recombination and replication have also been described for some KIR genes particularly those of the B haplotype (15–17). The CNV seems to influence the licensing of KIR<sup>+</sup> NK cells (18). Overall, various KIR genotypes can be observed in a population. All KIR genes, and especially for inhibitory KIR, a high degree of allelic polymorphism has been described. The latest KIR Immuno Polymorphism Database (IPD–KIR) describes 753 KIR alleles. KIR allele polymorphisms need to be investigated throughout the exon and the intron regions, and regulatory regions as shown for KIR3DL1 (19). In contrast to HLA class I genes, structure and length of KIR genes vary. KIR allele polymorphisms impact both KIR<sup>+</sup> NK cell phenotype and function, as we and other groups having described for KIR3DL1 (20–25) and for KIR2DL2/L3 (26). Differences in the intensity of expression (strong, weak, or null) have been described for the KIR3DL1 receptor, defining different allotypes according to the

KIR3DL1/3DS1 allele combinations present in healthy individuals (21, 27). Furthermore, the nature of KIR3DL1 alleles does not only impact the KIR3DL1 cell density but also the strength of the KIR3DL1–HLA interactions which in turn can affect NK cell functions (28, 29). The recognition of KIR allotypes using anti-KIR monoclonal antibodies also varies depending on the KIR allele polymorphism (30).

Taking these points into account, it is therefore necessary to thoroughly investigate the phenotypic and functional impact of KIR allele polymorphisms. Until now, potential KIR<sup>+</sup> NK cell alloreactivity in HSCT was mainly evaluated depending on the KIR/KIR ligand genetic combinations present and analyzed only at a generic level (i.e., presence or absence of KIR genes and KIR ligand). We speculate that KIR allele polymorphisms may alter donor KIR<sup>+</sup> NK cell phenotype/function, and thus modulate their alloreactivity affecting HSCT outcome. However, the impact of KIR allele polymorphisms on HSCT outcome remains difficult to assess due to the lack of suitable allele typing methods for all KIR genes. Until recently, several standard methods are used to type KIR genes at allelic level. Those methods include sequence-specific oligoprobe hybridization (31–37), sequence-specific primer (SSP) typing (22), SNP assay (38), Sanger sequence-based typing (SBT) (20, 39–42), high-resolution melting (43), and also combined SSP/SBT (21, 44). KIR allelic polymorphisms have been investigated for a few functional KIR genes (KIR2DL1/2DL2/2DL3/2DS1/3DL1/3DS1). Standard methods to type KIR genes at allelic level are usually single KIR locus specific and/or target a limited polymorphism. In addition, the constant increase in the number of KIR alleles described generates more and more ambiguous KIR typing in heterozygous samples since KIR polymorphism can extend over the entire gene. Recent advances in high-throughput sequencing technology [next-generation sequencing (NGS)], especially in immunology and hematology (45), enable determination of KIR alleles and KIR gene CNV. The extent of KIR allele polymorphisms, as demonstrated by exome capture, reported 37 new KIR alleles from 15 healthy South African individuals (46). Recently, whole KIR genome sequencing by NGS was used as a control method to validate CNV genotyping in the KIR locus (17). An exome capture that focused on KIR and HLA class I loci was also recently described (47). In this study, we developed a reliable NGS method for high quality DNA samples and easily implemented for the study of KIR allele polymorphisms.

## MATERIALS AND METHODS

### Samples

Thirty B-EBV cell lines from the 10th International Histocompatibility Workshop (IHW) were selected from a well-characterized panel known for their KIR gene content. KIR genotype information, including KIR allele typing of some KIR genes for all these B-EBV cell lines, was obtained either from the IPD/KIR database or from literature for specific KIR loci. Known KIR genotypes and allele typing of these 30 B-EBV cell lines are provided in the Table S1 in Supplementary Material.

## 215 KIR Long-Range (LR) PCR and Primers

216 DNA genomic extractions were performed from B-EBV cell lines  
217 using a Nucleospin blood kit (Macherey-Nagel, Duren, Germany).  
218 The concentration and the purity of all DNA samples were checked  
219 on a NanoDrop 2000C spectrophotometer (ThermoFisher,  
220 Wilmington, DE, USA) by measuring the ratio of absorbance  
221 at 260 and 280 nm. In parallel, 1.5 µg of each DNA sample was  
222 loaded on an agarose gel to check the DNA integrity. For KIR LR  
223 PCR, five intergenic KIR primers already described (17) and one  
224 additional *in-house* designed primer including four forward primers  
225 (#1, 5'-gccaataaacatcctgtgctgctcagct-3'; #2, 5'-ctcacaacatc-  
226 ctgtgtgctgctaactga-3'; #4, 5'-acggctgcctgtctgcacagacagcacc-3', #6,  
227 5'-cacatgctgcaccggctcagtcgagccga-3') and two reverse primers  
228 (#3, 5'-ttggagaggtggcaggggtcaagt-3'; #5, 5'-ctccatctgagggtccc-  
229 ctgaatgtg-3') were used to amplify the whole KIR genome.

230 The KIR LR-PCR protocol was optimized using the method  
231 described by Vendelbosch et al. (17). Briefly, KIR LR-PCR was  
232 performed with 2.5 U of PrimeSTAR GXL DNA Polymerase  
233 (Ozyme, Saint-Quentin en Yvelines, France), 1× PrimeSTAR  
234 GXL buffer, 200 µM of dNTP mixture (Ozyme) and 0.2 µM  
235 final concentration of each KIR primer. The LR-PCR reaction  
236 was performed in a C1000 Touch™ Thermal Cycler (Biorad,  
237 Marnes la Coquette, France) consisted of an initial denaturation  
238 of 2 min at 94°C followed by 30 cycles of 20 s at 94°C, 12 min  
239 at 68°C and 1 cycle of final elongation of 10 min at 72°C in the  
240 final 50 µL volume. This protocol enables amplification of each  
241 KIR gene from 5' to 3' untranslated regions (UTR). The final KIR  
242 LR-PCR product was run on 0.7% Seakem agarose gel in TBE1X  
243 (Lonza, Verviers, Belgium) and visualized by staining with the  
244 SYBR® safe (Invitrogen, Villebon sur Yvette, France) using the  
245 SimplyLoad™ Tandem DNA ladder size marker (Ozyme) to  
246 confirm the amplification and correct fragment size as well as to  
247 check for non-specific amplification.

## 249 Library Preparation and Sequencing

250 Qubit dsDNA High Sensitivity Assay Kit (Life Technologies,  
251 Villebon sur Yvette, France) was used to quantify the starting  
252 DNA library in the Qubit® fluorometer (Life Technologies). The  
253 library preparation was performed using the NGSgo GENDX  
254 kit (Bedia Genomics, Chavenay, France). To achieve the optimal  
255 insert size and library concentration, 250 ng of each genomic  
256 DNA was randomly fragmented according to the manufacturer's  
257 instructions. Briefly, 8.25 µL of NGSgo master mix (prepared from  
258 2 µL of NGSgo-LibrX Fragmentase buffer plus 3.25 µL of NGSgo-  
259 LibrX End Prep buffer plus 1.5 µL of NGSgo-LibrX Fragmentase  
260 Enzyme plus 1.5 µL NGSgo-LibrX End Prep Enzyme) (Bedia  
261 Genomics, Chavenay, France) was added to each genomic DNA  
262 in a final volume of 32.5 µL. The fragmentation, end-repair,  
263 and dA-tailing reactions were performed in a T100™ Thermal  
264 Cycler (Biorad, France) consisted of 20 min of fragmentation and  
265 end-repair at 25°C followed by 10 min of dA-tailing at 70°C. The  
266 dA-tailed DNA fragments of each sample were then subjected  
267 to adapter ligation in 9.25 µL of an NGSgo master mix contain-  
268 ing 7.5 µL of NGSgo-LibrX Ligase mix, 0.5 µL of NGSgo-LibrX  
269 Ligation Enhancer, 0.25 µL of NGSgo-Indx adapter for Illumina,  
270 and 1 µL of nuclease free water. The adapter ligation reaction took  
271 place in a T100™ Thermal Cycler (Biorad, France) for 15 min at

272 20°C followed by a cooling step at 15°C. The first cleaning and size  
273 selecting of the samples after adapter ligation were performed in  
274 a 0.45× beads:DNA ratio by using the Agencourt® Ampure XP  
275 (Beckman Coulter, Villepinte, France) according to the manufac-  
276 turer's instructions and eluted in 12.5 µL of 0.1× elution buffer  
277 (Lonza Rockland, USA). The size-selected, adapter-ligated DNA  
278 fragments of each DNA sample were then dual indexed with 15 µL  
279 of NGSgo reaction mix made from 12.5 µL of NGSgo-LibrX HiFi  
280 PCR mix plus 1.25 µL of NGSgo-Indx IN-5 and 1.25 µL of NGSgo-  
281 Indx IN-7 in a final volume of 25 µL followed by a PCR reaction in  
282 a T100™ Thermal Cycler (Biorad). PCR cycling was performed  
283 as follows: an initial denaturation of 30 s at 98°C followed by 10  
284 cycles of 10 s at 98°C, 30 s at 65°C, 30 s at 72°C and 1 cycle of final  
285 elongation step of 5 min at 72°C in the final volume of 25 µL. A  
286 second DNA cleaning and size selecting was performed in a 0.6×  
287 beads: DNA ratio by using the Agencourt® Ampure XP beads  
288 according to the manufacturer instructions and eluted in 16.5 µL  
289 of 0.1× elution buffer (Lonza Rockland, USA).

290 Quality control procedure for the library preparation included  
291 verification of fragment size before and after purification by using  
292 the QiAxcel Advanced System (QiAgen, Courtaboeuf, France).  
293 The pooled and barcoded libraries were denaturated with 0.2 M  
294 of NaOH and diluted in the pre-chilled HT1-buffer to obtain a  
295 final library concentration of 12 pM. The final denatured library  
296 was subsequently sequenced by using the MiSeq sequencer  
297 (Illumina, Biogenouest Genomics Platform Core Facility, Nantes,  
298 France; HLA Laboratory, EFS Nantes, France) with 500 cycles v2  
299 kits, which generated 250-bp end sequence reads.

## 301 Sequencing Data Analysis and KIR Allele 302 Assignment

303 The quality of the Illumina raw data sequences obtained was  
304 monitored by using the Sequencing Analysis Viewer Illumina  
305 software. The quality of the base calling from images and  
306 sequences was determined by the quality score (Q30) which must  
307 be ≥75% for 2 bp × 250 bp reads. KIR reads were mapped to the  
308 human genome reference sequence hg19 (GRCh37) by using the  
309 Burrows–Wheeler Aligner Memory Efficient Mapping (BWA-  
310 MEM) tool. The binary alignment map (BAM) files containing  
311 mapped reads were then visualized on Integrative Genomics  
312 Viewer (IGV) algorithm (48).

313 In parallel, raw KIR sequences were aligned and visualized  
314 using the Profiler software version 1.70, initially developed by  
315 Dr. M. Alizadeh (Research Laboratory, Blood Bank, Rennes,  
316 France) for NGS-based HLA typing (49). A flowchart for data  
317 analysis using the Profiler software is provided in Figure S1 in  
318 Supplementary Material. The first step of analysis consists by  
319 merging R1 and R2 sequences to each other when at least 10  
320 complementary bases were found between R1 and R2 of the  
321 same cluster. During this phase, for each inconsistency of base  
322 calling, the quality value was used to select the best assignment.  
323 All sequences issued from a cluster for which we could not deter-  
324 mine complementary between R1 and R2 remained unchanged.  
325 All sequences were transformed to FASTA format at the end of  
326 this step. The second step of analysis consists of phasing each of  
327 the sequences obtained in step one by using Blast algorithm. The  
328 third step of analysis consists by merging all sequences together

329 using Blast information. In this step, the depth for each position  
330 and the number of sequences for each allele were calculated.  
331 The first three steps are managed in a Linux environment. The  
332 last step is presentation and assignment of each construction  
333 based on database information in a friendly interface for user,  
334 all mismatches and differences to the database are extracted and  
335 presented to the user.

336 For KIR allele assignment, a manual bioinformatic pipeline  
337 was first used in the absence of available softwares. This consisted  
338 of exporting from IGV, all exon sequences of each KIR gene and  
339 comparing polymorphic bases with those referenced from the  
340 IPD–KIR database. Then, two different bioinformatics algorithms  
341 were used: the first one, hereafter called “BiRD,” was developed  
342 by the BiRD platform (E. Charpentier, U. Guyet, Genomics and  
343 Bioinformatics Core Facility GenoBiRD, Nantes, France) and  
344 consists of an analysis pipeline built with Snakemake on the same  
345 logic as the manual method. A flowchart for data analysis using  
346 the BiRD software is provided in Figure S2 in Supplementary  
347 Material.

### 349 Harvesting KIR-Specific Reads

350 First, raw sequences from fastq files are processed through  
351 cutadapt (v1.8.1) in order to remove Illumina adapter sequences.  
352 The cleaned reads are then mapped to hg19 (GRCh37) reference  
353 genome using BWA-MEM (v0.7.12) with the default parameters.  
354

### 355 Determining Presence/Absence of KIR Genes

356 Absence or presence of KIR genes is evaluated using GATK  
357 DepthOfCoverage on the BAM and using a browser extensible  
358 data (BED) file describing the chromosome position of each gene  
359 (except KIR2DP1 and KIR3DP1). Coverage mean is calculated  
360 on each gene position, and a threshold of 10 is applied in order to  
361 ascertain its absence or presence. Presence/absence of KIR genes  
362 defined by NGS is concordant to the KIR genotype of the 30 IHW  
363 samples, stratified by AA vs Bx genotypes, previously validated in  
364 our laboratory by PCR-SSP multiplex method (data not shown).  
365

### 366 Determining KIR Alleles

367 Allele-specific nucleotide positions are extracted manually using  
368 IPD–KIR alignment tool.<sup>1</sup> For every gene, the Nucleotide—CDS  
369 of all alleles are aligned against the default reference allele. A  
370 python script is then used to reformat the multipage alignments  
371 in order to have one allele alignment per line. A second python  
372 script is utilized to extract all variations from the default reference  
373 allele and map the exon position number of these variations to  
374 the chromosome position. A file is created for each gene listing  
375 all the variations found for every allele. Bases at these positions  
376 are then called using SAMTools (v1.2-2) mpileup for all samples.  
377 Finally, KIR alleles are determined by calculating the percentage  
378 of nucleotide matches between the base calls and the allele varia-  
379 tions for each KIR allele, the highest percentage giving the most  
380 confident allele.

381 The second algorithm used for KIR allele assignment was  
382 the Profiler software, previously described in Figure S1 in  
383 Supplementary Material, version 1.70 (49), which permits to  
384

385 <sup>1</sup><https://www.ebi.ac.uk/ipd/kir/align.html>.

386 directly assign KIR alleles at the highest level resolution (seven  
387 digits) since full intron and exon sequences were considered and  
388 also provides quality data such as mean coverage for each KIR  
389 locus. The fragment size percentage of sequences for each allele/  
390 locus was also considered as well as percentage of mapping for  
391 each KIR gene.

392 Overall, KIR allele assignment for each locus and for all  
393 samples corresponds to the combined KIR results obtained using  
394 manual pipelines, BiRD, and Profiler softwares. KIR alleles were  
395 assigned on the basis of the known DNA sequences identity within  
396 the IPD/KIR database.<sup>2</sup> KIR alleles are named in an analogous  
397 fashion as the nomenclature used for HLA class I alleles. After the  
398 gene name, an asterisk is used as a separator before a numerical  
399 allele designation. The first three digits of the numerical designa-  
400 tion are used to indicate alleles that differ in the sequences of  
401 their encoded proteins. The next two digits are used to distinguish  
402 alleles that only differ by synonymous (non-coding) differences  
403 within the coding sequence. The final two digits are used to  
404 distinguish alleles that only differ by substitutions in an intron,  
405 promoter, or other non-coding region of the sequence.  
406

## 407 RESULTS

### 409 LR KIR Gene Amplifications

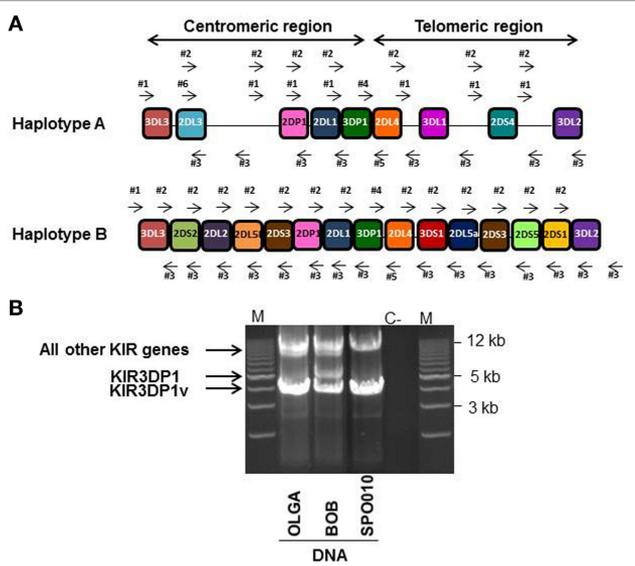
410 Thirty reference IHW samples with known KIR genotyping  
411 (Table S1 in Supplementary Material) were used to validate our  
412 NGS method for typing of each KIR gene at allelic resolution.  
413 DNA integrity, checked by loading each sample on an agarose  
414 gel, confirmed high quality for all samples (data not shown). In  
415 order to amplify all KIR genes from the 5′ UTR to the 3′ UTR,  
416 six intergenic KIR primers were chosen to allow the amplifica-  
417 tion of framework KIR genes. These intergenic primers also  
418 amplify KIR genes located either in the centromeric or telomeric  
419 region, which belong to the A and/or B specific KIR haplotype  
420 genes (Figure 1A). A robust LR amplification of KIR genes was  
421 obtained for all samples as illustrated for three representative  
422 IHW samples (Figure 1B). One specific band between 4 and 5 kb  
423 for the KIR3DP1 pseudogene and another specific band between  
424 9 and 17 kb corresponding to a cluster of all other KIR genes were  
425 observed, irrespective of KIR AA or AB genotype (Figure 1B) as  
426 KIR genomic length varies depending on KIR genes (Table S2 in  
427 Supplementary Material). For some IHW samples such as BOB,  
428 two specific bands at 4 and 5 kb were observed for the KIR3DP1  
429 gene corresponding to KIR3DP1\*003 and KIR3DP1\*001  
430 variants, respectively, whereas only one band at 4 kb specific  
431 of KIR3DP1\*003 variant was observed for OLGA and SPO10  
432 samples (Figure 1B).  
433

### 434 Complete Sequencing of All KIR Genes

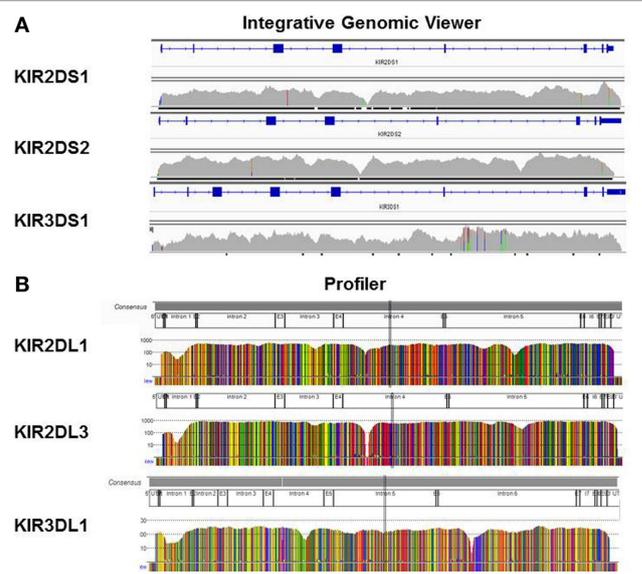
435 In order to check the specificity of KIR LR-PCR obtained,  
436 amplicons were further fragmented and sequenced on paired  
437 end 2 bp × 250 bp from Illumina MiSeq platform. The sequenc-  
438 ing of all amplicons yielded a total of 6.3 Gb, which was gener-  
439 ated from a  $755 \pm 31$  K/mm<sup>2</sup> cluster density (data not shown).  
440 Approximately 88.2% of the clusters passed QC filters and on  
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442 <sup>2</sup><http://www.ebi.ac.uk/ipd/kir/alleles>.

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**FIGURE 1 | Long-range (LR) killer immunoglobulin-like receptor (KIR) gene amplification. (A)** Six intergenic KIR primers (four forward primers: #1, #2, #4, and #6 and two reverse primers: #3, and #5) were used to perform LR PCR amplifications. These primers were able to amplify full length KIR genes in both the centromeric and telomeric regions belonging either to the A or B KIR haplotype. **(B)** Illustrative 0.7% agarose gel electrophoresis of LR PCR amplifications spanning the KIR genome of three representative International Histocompatibility Workshop (IHW) DNA samples. One IHW sample with an AA KIR genotype (i.e., SPO010) and two DNA samples with an AB KIR genotype (i.e., OLGA and BOB) were used. Amplicon lengths vary from 4 to 5 kb for the KIR3DP1 gene to 9–17 kb for all other KIR genes. Two specific bands of 4 and 5 kb corresponding to two KIR3DP1 variants were observed for BOB sample. M: Tandem ladder Lonza Seakem size marker; C: H<sub>2</sub>O, negative control.



**FIGURE 2 | Full sequencing of all killer immunoglobulin-like receptor (KIR) genes.** Reads were mapped to the human genome reference sequence hg19 using the Burrows–Wheeler Aligner Memory Efficient Mapping tool. The binary alignment map files containing mapped reads were then visualized on the Integrative Genomics Viewer as illustrated for KIR2DS1, KIR2DS2, and KIR3DS1 genes **(A)** or using Profiler software **(B)** as illustrated for KIR2DL1, KIR2DL3, and KIR3DL1 genes from one representative International Histocompatibility Workshop DNA sample.

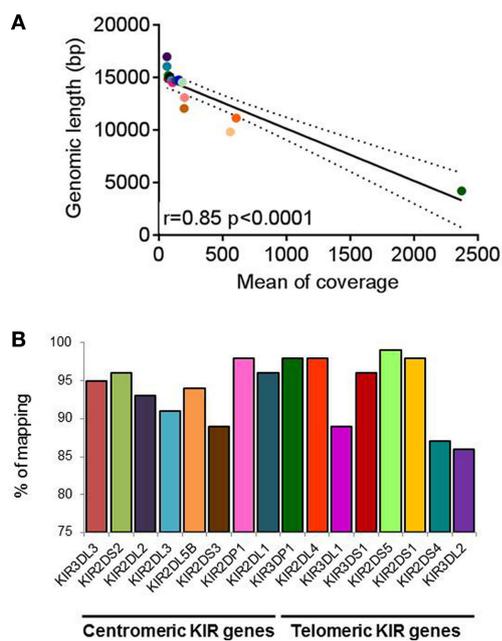
average, 82.4% of both reads passed with a Q30 > 82% (data not shown). Thus, analysis of FastQ data obtained from all IHW samples reported an excellent quality control. The entire length of KIR genes was sequenced with good coverage as illustrated for KIR2DS1, KIR2DS2, and KIR3DS1 (Figure 2A) activating genes, and for KIR2DL1, KIR2DL3, and KIR3DL1 (Figure 2B) inhibitory genes using either IGV or Profiler software, respectively. For all genes, the depth of coverage varies most at the beginning and at the end of the amplicons, but all key regions were sufficiently covered. In particular, we observed that mean coverage ranged from 62.5× (KIR2DS4) to 2,373.3× (KIR3DP1) leading to a mean coverage of 316.55× for all KIR genes except for KIR2DL5A genes since not analyzed using Profiler (Table S3 in Supplementary Material). A significant correlation was observed between mean coverage and genomic KIR length ( $r = 0.85, p < 0.0001$ ) as illustrated Figure 3A. Indeed, the lower the genomic length, the higher the mean coverage is as illustrated for the KIR3DP1 gene. The mean percentage of mapping, established by the coverage of amplicon, ranged from 86.2% (KIR3DL2) to 98.2% (KIR2DP1 and KIR3DP1) (Figure 3B; Table S3 in Supplementary Material) suggesting that sufficient read depth was obtained for determination of all KIR genes. However, KIR2DL5A reads could have been mapped

only using BWA-MEM software and BiRD algorithm. Overall, these results demonstrate the efficiency of our NGS-KIR allele typing approach to capture the full KIR genomic locus and the uniformity of coverage for each KIR locus confers assurance for KIR allele assignment.

### Specificity of NGS-Based KIR Allele Typing

Due to the high degree of KIR polymorphisms and the fact that NGS technology generates a lot of sequencing reads, three different algorithms were evaluated to increase the reliability of KIR allele assignment as reported for NGS-based HLA typing (50). KIR allele assignment was first done manually and then confirmed using both BiRD pipeline and Profiler software. Overall, resulting KIR allele assignments of the 30 reference IHW samples were feasible for all loci and for the majority of samples without remaining ambiguities (Table 1).

We further evaluated the strength of our NGS-based method for KIR allele assignment. For all IHW reference samples tested ( $N = 30$ ), the number of KIR alleles previously known in the IPD/KIR database and those obtained by our NGS-KIR based typing approach was compared for each KIR locus. As an example, from the 30 IHW samples tested, only 5 KIR3DL3 alleles out of 60 expected alleles for this framework gene were previously known in the IPD/KIR database (Table S1 in Supplementary Material), 54 KIR3DL3 alleles from 24 heterozygous and 6 homozygous samples (Table 1) were assigned



**FIGURE 3 | Coverage and percentage of mapping obtained for killer immunoglobulin-like receptor (KIR) genes. (A)** Correlation graph representing mean coverage for each KIR gene and KIR genomic length. Mean coverage was estimated for each KIR gene present for all International Histocompatibility Workshop (IHW) samples using Profiler software. Statistical significance was determined using the Pearson's rank coefficient using GraphPad Prism version 6 software (GraphPad Software, La Jolla, CA, USA). A significant  $p$ -value between mean coverage and genomic KIR length was observed ( $p < 0.0001$ ). **(B)** Bars representing the percentage of mapping of each centromeric and telomeric KIR gene present for all IHW samples determined using Profiler software. KIR2DL5A locus was not included since not analyzed using Profiler.

by our NGS-based KIR allele typing approach (Figure 4). Our NGS-based KIR allele typing approach permits identification of additional framework KIR alleles, e.g., KIR3DP1 ( $n = 43$ ), KIR2DL4 ( $n = 48$ ), and KIR3DL2 ( $n = 44$ ) (Table 1; Figure 4). NGS-based KIR typing method also allows the identification of polymorphisms of well-functionally characterized KIR by increasing the number of assigned KIR alleles of the 30 IHW samples available in the IPD/KIR database (Table S1 in Supplementary Material), e.g., KIR2DL1 ( $n = 30$ ), KIR2DL2 ( $n = 10$ ), KIR2DS1 ( $n = 11$ ), KIR2DS2 ( $n = 12$ ), KIR3DL1 ( $n = 35$ ), and KIR3DS1 ( $n = 11$ ) (Table 1; Figure 4). The number of activating KIR2DS1, KIR2DS2, and KIR3DS1 assigned alleles by NGS remained low because only IHW samples with the corresponding activating KIR gene were included in this analysis. Overall, a higher number of KIR alleles were identified from these 30 IHW samples by our shotgun NGS methodology compared to those previously characterized by other less sensitive methods, as referred to in the IPD/KIR database ( $N = 422$  vs  $N = 233$ , respectively).

The knowledge of KIR allele typing of IHW samples, recently updated thanks to an exome capture (47), permits to evaluate the concordance of our NGS-based KIR allele results (Table 1) with

those of Norman et al. since 22 IHW samples were commonly used in both methods (Table S1 in Supplementary Material). In this case, a large number of allelic KIR typing for all loci was compared ensuring the reliability of our NGS-based KIR allele typing method. For each KIR locus and for the 22 IHW concerned samples, KIR allele typing results were divided into: concordant (one KIR allele matched for homozygous samples or two KIR alleles matched for heterozygous samples), semi-concordant (one KIR allele matched and one KIR allele mismatched), and discordant (one KIR allele mismatched for homozygous sample or two KIR alleles mismatched for heterozygous sample). For each KIR allele, only the first three digits were taken into account for the assessment of concordance. Complete concordance (100%) of KIR allele typing was demonstrated in 11 KIR genes. The concordant genes were KIR2DS2 (8 samples out of 8), KIR2DL5B (2 out of 2), KIR2DS3 (1 out of 1), KIR2DL1 (18 out of 18), KIR2DL4 (22 out of 22), KIR3DL1 (17 out of 17), KIR3DS1 (7 out of 7), KIR2DL5A (7 out of 7), KIR2DS5 (6 out of 6), KIR2DS1 (6 out of 6), and KIR2DS4 (16 out of 16) (Figure 5). Concordant results were observed, but at a lesser frequency for KIR3DL3 (20 out of 22, i.e., 91%), KIR2DL2 (4 out of 5, i.e., 80%), KIR2DL3 (7 out of 8, i.e., 88%), KIR2DP1 (16 out of 20, i.e., 80%), KIR3DP1 (16 out of 18, i.e., 89%), and KIR3DL2 (16 out of 18, i.e., 89%) (Figure 5).

Ten semi-discordant KIR allele results and two discordant KIR allele results between our NGS-based method and exome data were identified (Table 2). Except for the pseudogene KIR2DP1, with four IHW samples, these discrepancies were limited to 1 or 2 out of 22 IHW samples per locus (Table 2). KIR allele determinations using manual, BiRD algorithm, and different versions (the latest one Rev 2.0.188) of Profiler software were carefully reviewed. Only IHW samples sequenced on different runs and with the same KIR allelic results were reported (data not shown). These potential discrepancies (5%), possibly linked to the design and implementation of each algorithm, need to be further validated by another typing method such as SSP or sequencing.

Overall, our NGS-based method and exome data showed a rate of concordance of 95% for all loci, established for all KIR genes on 22 IHW samples, suggesting a reliable method.

## DISCUSSION

In this study, we developed an NGS-based KIR allele typing approach to characterize the sequence of all polymorphic KIR genes. Our method of typing all KIR genes at high resolution provides an alternative, easily implemented method practice, to study the KIR allele polymorphisms. It may be a cheaper method than exome capture (47). This tool is currently adapted to the KIR gene large-scale analysis. Using our approach, the majority of KIR alleles previously uncharacterized by standard methods were clearly identified from genomic DNA of 30 B-EBV cell lines from the 10th IHW. High quality DNA samples, high fidelity of enzyme polymerase, and a reliable library preparation were needed since evaluation of different Taq polymerase enzymes

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**TABLE 1 | Next-generation sequencing-based killer immunoglobulin-like receptor (KIR) allele typings of 30 reference B-EBV cell lines from the 10th International Histocompatibility Workshop.**

ID	Centromeric KIR genes										Telomeric KIR genes									
	3DL3	2DS2	2DL2	2DL3	2DL5B	2DS3	2DP1	2DL1	3DP1	2DL4	3DL1	3DS1	2DL5A	2DS3	2DS5	2DS1	2DS4	3DL2		
AMAI	*013 *041		+				*00301 *004	+	*003 *006	*0080101 *0080102	*001					*00301		*00101		
AMALA	*0402 *00802	*00101	*00301	*001			*00201	*00302	*007 *00901	*00102 *00501	*01502		*001		*00201	*00201	*001	*0020105 *0070102		
BOB	*00101 *01303	*00101	*00301	*00201			*00301	*00302	*002 *00302	*001 *005	*002 *001301	*01301	*00101		*00201	*00201	*001	*0020101 *0070102		
BRIP	*00801	*00104 *004	*00301	+		*00103 *00201	*0010201 *0020101	*00302	+	*0010305 *00501	*008	*01301	*00103 *00501	*00103 *00201	*002	*003	*003	*0070102 *0070103		
CALOG ERO	*00207 *01001		+				*00201	*00302	*00302 *010	*008	*001 *004					*00301 *00601		*00101 *00301		
COX	*00102 *00103	*00102	*00201 *007				*00301	*00201	*005 *006	*00501* *011	*005010	*055	*00101		*00201	*00201	*010	*00103 *007		
DEU	*00101 *01402	*00101	*00201				*00301	*00201	*001 *006	*00801 *011	*00101 *00501					*003 *010	*01001 *01101			
DKB	*00101 *006		+				*00301	*00201	*00302 *006	*0010201 *00103	*002 *02001					*00101	*0020101 *00902			
HC301	*014	*00101 *002	*00101 *00301		*010	*00103 *00201	*00102	*004 *010	*003010 *004	*00102	*002			*00103 *00201		*001	*001	*00201		
HID	*01402 *018		*00101				*00201	*00302	*00302 *010	*00102	*01502					*00101		*00201		
HOM-2	*00101 *0090101		+				*00201	*00302	*00302 *006	*00801 *00802	*001 *004					*00301 *00601	*0010102 *00501			
HOR	*001 *048		+				*00301	*00201	+	*00501		*01301	*00101		*002	*00201		*007 *021		
JHAF	*00901 *026		*00101				*002	*00302	*00302	*011	*00501					*010		*001 *01001		
JVM	*007 *00801	*00101	*00301	+			*005	*00302	*001 *00302	*00103 *00801	*00101 *008					*003010		*00101 *009		
KAS011	*00901 *01302		+				*002	*00201	*00302 *006	*00103 *005	*008	*01301	*00101		*00201	*00301		*01001 *019		
KAS116	*013 *01501		+				*002	*00302	+	*011	*00501					*010		*0103 *010		
LBUF	*00301 *0090101		+	*001			*002	+	*00302 *0090102	*00102 *011	+				+			+		
LUY	*001 *02701		*00101 *00501				*00201 *00301	*00302	*00302	*00801 *011	*00401 *00501					*00601 *010		*001 *00501		
MOU	*00207 *00801		*001				*00201 *005	*00302	*00302	*00801	*00101 *00401					*00301 *00601		*010 *01101		

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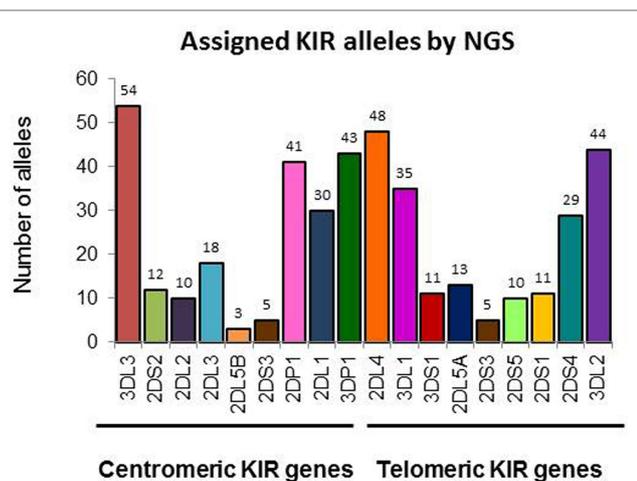
**TABLE 1 | Continued**

ID	Centromeric KIR genes										Telomeric KIR genes									
	3DL3	2DS2	2DL2	2DL3	2DL5B	2DS3	2DP1	2DL1	3DP1	2DL4	3DL1	3DS1	2DL5A	2DS3	2DS5	2DS1	2DS4	3DL2	3DL2	
OLGA	*00201 *00902		*00101	*00101			*00201 *006	*00302	*00302	*005 *011	*001 *00501	*01301 *01301	*00103		*002	*002	*010	*00701		
PE117	*00101 *01002		*00101 *00201				*00201 *00301	*00201 *00302	*00901	*00501 *00802	*00401 *00101				*00201	*00201	*00601	*00701 *018		
PF04015	*01402	*00101	*00101 *003						*001	*011	*00501						*010	*00103		
RSH	*0040202 *00901	*00101	+	+	*004		*00201 *009	*00302 *01201	*00304 *008	*0010307 *011	*00501 *017			*006		+	+			
SAVC	*00801 *00202		*00101				*008 *00201	*00302	*00302	*00102 *00802	*00401 *01502						*006010	2*00202 *00301		
SPO010	*00206		+				*00201	*00302	+	*011	*0050101						*010	*001		
T7526	*0090101		*00101				*00201	*00302	*00302	*00501 *00102	*01502	*013	*00101		*00201	*002	*001	*0020105 *0070102		
VAVY	*002 *017		*00101 *00201				*002 *003	*002 *00302	*00302 *006	*011	*00501						*010	*0010302		
WT51	*00103 *036	*00101	+	+	*00201	+	*001 *004	+	+	*00501	*01301	*00101 *00501			*002	*002	+	+		
WDV	*00301 *0090101	*00101	*003	+		*002	*002	*00302 *00901	*00302	*00501	*01301	*00501	*002			*00201		*0070103		
YAR	*00102		+	+			*002 *003	+	*00302 *006	*0010201 *011	+					+	+	+		

Results are presented according to the centromeric or telomeric localization of KIR genes on human genome. KIR alleles were named according to the last nomenclature available on the IPD/KIR database (<http://www.ebi.ac.uk/ipd/kir/>). + indicates the presence of a specific KIR gene. ID, sample identification.

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**FIGURE 4 | Number of killer immunoglobulin-like receptor (KIR) allele assigned by next-generation sequencing (NGS).** Number of KIR alleles assigned for each centromeric and telomeric KIR gene of 30 International Histocompatibility Workshop samples obtained by our NGS method. KIR allele assignment for each locus and for all samples corresponds to the combined KIR allelic results obtained using manual pipelines, BiRD, and Profiler software.

and library preparation kits gave conflicting results (data not shown).

Our study evaluated the performance of different algorithms for KIR allele assignment. Reliability of the manual, BiRD pipeline, and Profiler software was tested since neither algorithm alone was able to provide 100% accuracy for all KIR loci. Our results showed that the Profiler software was reliable to assign KIR alleles through the full length of each KIR gene, excluding KIRDL5A variants. In this case, KIR2DL5A and KIR2DL5B sequences were too closed, and Profiler software failed to accurately analyze both sequences. Since all coding, non-coding, and regulatory regions were explored, one could expect that a lot of new KIR alleles will soon be described. Analysis with Profiler consists of two distinct parts. The first part is performed in three steps in a Linux environment: the first step corresponds to the merging of each R1 and R2 issued from the same cluster to each other, each time that a complementarity of at least 10 bases is found, with correction or base calling inconsistencies using a quality value for each nucleotide. There are two interests in this step: longer sequences and lower sequences number were analyzed. The second step corresponds to the phasing of each sequence based on KIR databases using Blast algorithm. Third, the data file from Blast was used to merge all sequences together to construct each allele. In this step, calculation for depth of each position and the number of sequences used for each allele are determined. The second part is done on a Windows environment. A friendly interface presents graphics of all sequences for all studied loci. Assignment of all sequences is done using a database, highlighting all mismatches compared to reference and also differences between KIR alleles selected. Each allele is scored for quality control as per the European Federation for Immunogenetics guideline.

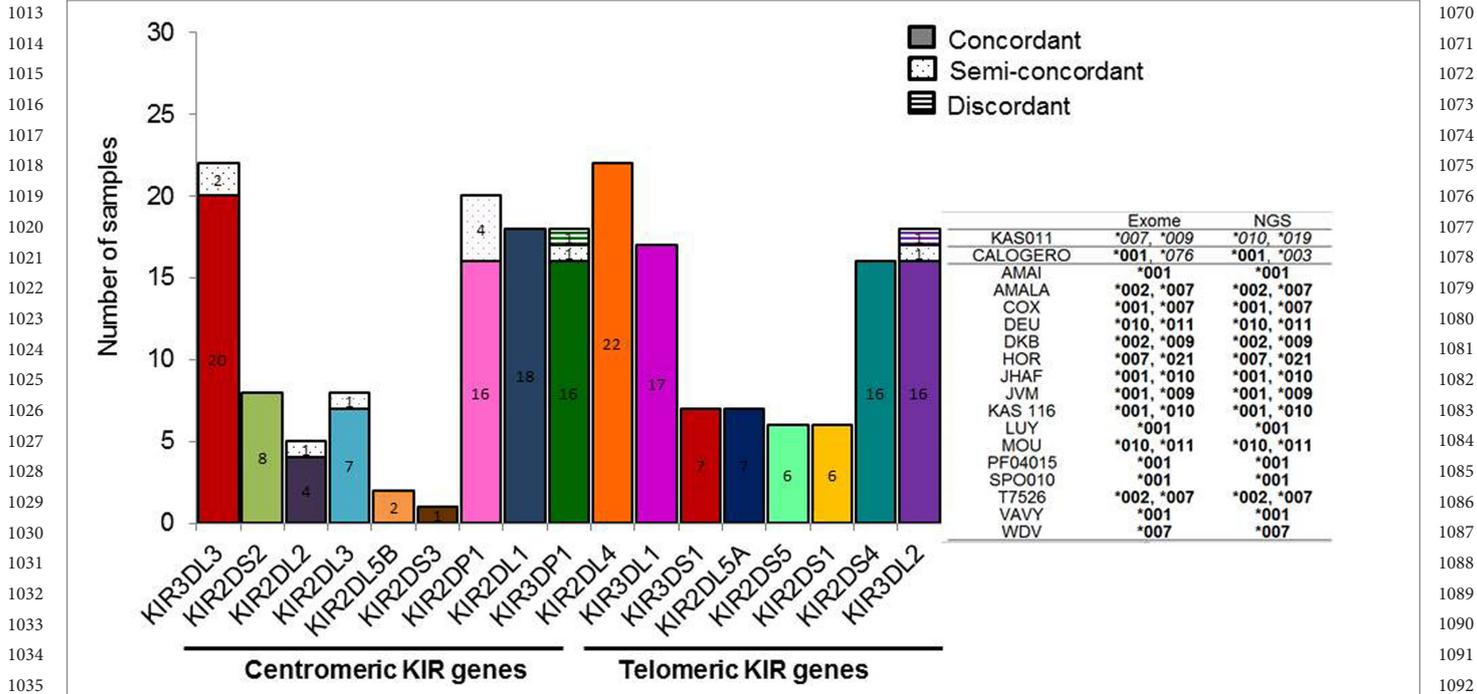
Killer immunoglobulin-like receptor alleles of all genes including KIR2DL5A, but excluding the pseudogenes KIR2DP1 and KIR3DP1, were assigned using BiRD algorithm. However, many allelic ambiguities remained when this pipeline was used alone (data not shown). It is likely that this is due to the fact that only coding regions (CDS) were taken into account for allele variation comparison. Analysis of all exon/intron polymorphisms, CNV detection, summary statistics of call accuracy for KIR gene content (presence/absence) and for KIR allele identification needs to be completed. Furthermore, the two pseudogenes KIR2DP1 and KIR3DP1 could be manually added to the BED file describing the gene positions on the genome in order to include them in the analysis pipeline.

Due to the time-consuming nature of manual KIR allele assignment, two different algorithms are needed to ensure the reliability of NGS-based typing methods for the identification of KIR allele polymorphisms.

Until now, KIR genetic population studies have often been restricted to the identification of KIR gene content, or of A and/or B KIR haplotypes (51, 52). Determination of KIR alleles in healthy individuals of a given population may provide a better definition of KIR haplotypes (52) and KIR gene linkage disequilibrium (53) and will considerably increase the IPD/KIR database.

The implementation of our suitable NGS.KIR method will enable analysis of all allelic polymorphism within KIR genes extending to all coding, non-coding, and regulatory regions. A link between KIR allelic polymorphism and the expression level and/or function of the corresponding KIR<sup>+</sup> NK cells is necessary for all KIR genes as previously established for the expression level of HLA-A and HLA-Cw molecules (54–56). We speculate that KIR allelic polymorphisms may affect not only the distribution and function of these gene products but also the licensing of NK subpopulations as described for HLA class I molecules (57, 58). Deep analysis of KIR<sup>+</sup> NK cell phenotype and function depending on KIR and HLA class I alleles present is needed to assess the diversity of KIR<sup>+</sup> NK cell repertoire (21, 59), as well as the specificity of anti-KIR antibodies (30, 60). Overall, the analysis of KIR allelic polymorphisms combined with the autologous HLA class I environment will enable better evaluation of KIR<sup>+</sup> NK cell functional subpopulations (61). This functional KIR<sup>+</sup> NK cell repertoire will be better defined by taking into account the nature of KIR alleles present in addition to the autologous HLA class I environment.

Investigation of KIR allelic polymorphism may be of an immunological interest in the context of viral infections such as those related to CMV (62), HIV (63), HCV (64), and of human reproduction (65). In the context of HSCT, inclusion of KIR allele typing in addition to HLA typing may provide a better evaluation of HSC donor's KIR<sup>+</sup> NK cell repertoire (21, 59, 60, 66, 67). An identification of those with the best antileukemic potential will provide a potential tool to determine an early posttransplant hematopoietic chimerism when donor and recipient have identical KIR genotypes (68) as well as the impact of KIR<sup>+</sup> NK cell alloreactivity on HSCT outcome (69–73). The functional relevance of typing both KIR and HLA genes at



**FIGURE 5 | Specificity of next-generation sequencing (NGS)-based killer immunoglobulin-like receptor (KIR) allele typing.** Bars representing the numbers of KIR allele typing obtained by our NGS-based method from 22 International Histocompatibility Workshop (IHW) samples compared with those assigned by exome analysis (47). In this case, KIR allele typing for each locus and for all samples corresponds to the combined KIR allelic results obtained using manual pipelines, BIRD, and Profiler softwares. Each bar represents one specific centromeric or telomeric KIR gene. Concordant (one KIR allele matched for homozygous typing or two KIR alleles matched for heterozygous typing), semi-discordant (one KIR allele mismatched for heterozygous typing), and discordant KIR allele typing (one KIR allele mismatched for homozygous typing or two KIR alleles mismatched for heterozygous typing) were highlighted by a specific color code. Representative KIR3DL2 typing of IHW samples obtained by exome analysis compared to those assigned by NGS method is provided in the right of the graphs. Concordances are highlighted in bold and discordances in italics.

**TABLE 2 | Discordant killer immunoglobulin-like receptor (KIR) typing of International Histocompatibility Workshop samples observed between typing obtained by exome capture<sup>a</sup> (47) and those obtained by next generation sequencing (NGS) in this study.<sup>b</sup>**

ID	Centromeric KIR genes						Telomeric KIR genes					
	3DL3		2DL2		2DL3		2DP1		3DP1		3DL2	
	Exome KIR typing <sup>a</sup>	NGS typing <sup>b</sup>	Exome KIR typing <sup>a</sup>	NGS typing <sup>b</sup>	Exome KIR typing <sup>a</sup>	NGS typing <sup>b</sup>	Exome KIR typing <sup>a</sup>	NGS typing <sup>b</sup>	Exome KIR typing <sup>a</sup>	NGS typing <sup>b</sup>	Exome KIR typing <sup>a</sup>	NGS typing <sup>b</sup>
AMAI							<i>*003, *013</i>	<i>*00301, *004</i>				
CALOGERO	<b>*00207</b> , <i>*017</i>	<i>*00207, *01001</i>							<i>*002, *00302</i>	<i>*00101, *076</i>	<i>*00101, *00301</i>	
COX					<b>*002</b>	<i>*00201, *007</i>						
DKB									<i>*015, *00302, *006</i>			
KAS011							<b>*002</b>	<i>*002, *00301</i>			<i>*00701, *01001, *00902, *019</i>	
LUY							<i>*002, *016</i>	<i>*00201, *00301</i>				
PF04015			<b>*003</b>	<i>*00101, *003</i>								
WT51							<i>*004, *018</i>	<i>*004, *001</i>				
YAR	<b>*00102</b> , <i>*044</i>	<b>*00102</b>										

Discrepancies are shown in italics for each KIR locus concerned. Allelic typing in bold represent concordant alleles. KIR alleles were named according to the last KIR nomenclature. ID, sample identification; “-” indicates absence of KIR gene.

1127 high resolution may help determine their combined effects on  
1128 outcome of HSCT.

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## 1131 AUTHOR CONTRIBUTIONS

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1133 BM performed KIR allele typing by next-generation sequenc-  
1134 ing, KIR allele assignment, interpretation of data, and wrote the  
1135 manuscript. NL performed DNA extractions from EBV-B cell  
1136 lines, KIR genotyping and KIR allele typing by next-generation  
1137 sequencing and commented on the manuscript. MA upgraded  
1138 Profiler software integrating a KIR module, analyzed data, and  
1139 commented on the manuscript. UG developed a pipeline for  
1140 KIR allele assignment, analyzed data, and commented on the  
1141 manuscript. CW and GD performed DNA extractions from  
1142 EBV-B cell lines and commented on the manuscript. EC  
1143 supervised the development of a pipeline for KIR allele assign-  
1144 ment, provided a bioinformatic help on KIR read mapping,  
1145 and commented on the manuscript. AW provided advices on  
1146 the library construction setting and commented on the manu-  
1147 script. CR designed the study, analyzed and interpreted data,  
1148 commented on the manuscript, and contributed to writing the  
1149 manuscript. KG designed the study, analyzed data, and wrote  
1150 the paper. All the authors have approved the manuscript for  
1151 publication.

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## SUPPLEMENTARY MATERIAL

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1409 ducted in the absence of any commercial or financial relationships that could be  
1410 construed as a potential conflict of interest.
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