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Somatic Genomic Alterations in Haematological Tumours Can Interfere With Accurate HLA and Chimerism Diagnostics

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ABSTRACT

Tumour cells, which are often found in the peripheral blood of patients with acute leukaemia, may harbour multiple somatic alterations throughout the genome, including changes in the HLA region and short tandem repeat (STR) regions. We investigated whether such somatic alterations interfere with HLA and chimerism diagnostics conducted in preparation for an allogeneic haematopoietic stem cell transplantation (allo-HSCT). This study describes 10 patient-based cases for which laboratory diagnostics were performed prior to a possible stem cell transplant in the Maastricht University Medical Center. In three acute leukaemia patients, somatic alterations were detected within the HLA region in peripheral blood samples: one case showed a complete loss of an HLA haplotype, while two cases exhibited somatic mutations affecting a single HLA class I gene. Additionally, seven patients with haematological malignancies revealed somatic variations within the STR regions, indicated by the presence of a third allele or the partial or complete loss of an allele in pre-transplant peripheral blood samples. In all patients, these somatic variations were confirmed by repeating the tests using buccal swab samples from patients or samples from family members. Furthermore, our study demonstrated that somatic alterations within STR regions used for chimerism testing occurred in 6% of the 176 patients who received an allo-HSCT between 2017 and 2022. This study underscores the clinical relevance of detecting somatic alterations prior to allo-HSCT, as they may interfere with HLA and STR analysis, potentially leading to HLA mistyping or incorrect chimerism detection. Additionally, it highlights the frequency with which genetic changes in tumour cells can affect chimerism diagnostics. The findings emphasise the vital importance of selecting the appropriate sample source for typing purposes and considering the patient's karyotype when choosing STRs, especially when tumour cells are present in the peripheral blood of patients with haematological malignancies.

1 | Introduction

The intricate molecular mechanisms facilitating tumour cells to escape the host immune response are a characteristic feature of cancer, known as immune evasion. Central to this phenomenon is the downregulation of HLA molecules, essential for presenting peptides on the cell surface. Tumour-specific mutations can lead to the formation of neoantigens, which peptides are

Abbreviations: AML, acute myeloid leukaemia; ALL, acute lymphoblastic leukaemia; Allo-HSCT, allogeneic haematopoietic stem cell transplantation; CMML, chronic myelomonocytic leukaemia; HSCT, haematopoietic stem cell transplantation; MDS, myelodysplastic syndrome; RFU, relative fluorescent units; SSO, sequence specific oligonucleotide; STR, short tandem repeat.

subsequently presented in HLA class I molecules. Next, cytotoxic T-cells can target and kill these tumour cells due to the lack of central tolerance for these neoantigens [1]. However, tumour cells often evade immune surveillance by downregulating or losing HLA class I expression, making them less visible or invisible to T-cells. This downregulation is widespread, observed in up to 90% of human tumours, which frequently correlate with a poor prognosis [2, 3].

Genomic alterations, such as somatic mutations or deletions, are the key mechanisms that can result in the loss of one or several HLA genes or an entire HLA haplotype [4]. Notably, the loss of an HLA haplotype emerges as a critical factor during relapse after haematopoietic stem cell transplantation (HSCT), especially when an HLA-mismatched donor is involved [5–7]. Detecting HLA haplotype loss is crucial for tailoring effective post-HSCT relapse treatments and for selecting alternative donors for subsequent transplantation. Despite the well-established role of HLA loss in the post-HSCT setting, little is known about the occurrence of somatic HLA loss in patients at diagnosis, particularly in the pre-transplant phase. Although instances of HLA heterozygosity loss before HSCT have been reported [8–16], understanding how somatic changes impact HLA testing and subsequent donor selection remains of clinical relevance.

In the context of HSCT and chimerism diagnostics, the analysis of short tandem repeats (STRs) plays a pivotal role. STRs, which are microsatellites consisting of 2–6 nucleotide repeat units, constitute about 3% of the human genome and serve as key markers for engraftment monitoring in HSCT [17, 18]. Due to their trans-slippage replication mechanism, STRs exhibit high mutation rates, and the compromised mismatch repair system in malignant cells leads to microsatellite instability and increased mutation rates [19].

To our knowledge, only two studies have reported cases where alterations in STRs have affected the diagnostics for donor engraftment analysis. Pereira et al. reported five cases of STR alteration after transplantation; Three patients with acute myeloid leukaemia (AML) showed the loss of an informative recipient-specific allelic peak in a post-transplant sample due to loss of heterozygosity of these specific STRs. Additionally, in one AML patient, a mutational increase in the size of an STR was identified in a post-transplant sample [16]. Lin et al. described four cases in which chromosomal gain or loss was detected using STR analysis of samples from patients diagnosed with AML, myelodysplastic syndrome (MDS) or hepatosplenic T-cell lymphoma. They demonstrated the use of these STRs as markers of impending relapse after HSCT [20]. Currently, nothing is known about the occurrence of somatic mutations in the STRs in pre-transplant samples. This gap in our understanding emphasises the need for further research in this area.

In this study, we examined the impact of somatic alterations on HLA typing and chimerism testing conducted prior to HSCT. Our data contribute to the existing knowledge on the clinical relevance of correct diagnostic strategies in the pre-transplant setting, emphasising the potential adverse effects of somatic alterations.

2 | Materials and Methods

2.1 | Patients and Ethics

The 10 cases described in this study are based on the laboratory diagnostics performed for patients with AML, MDS, chronic myelomonocytic leukaemia (CMML) or acute lymphoblastic leukaemia (ALL) who were eligible for a stem cell transplant between 2011 and 2022 at the Maastricht University Medical Center+ (Table 1). To assess the occurrence of somatic variation within STR regions at the time of diagnosis, a cohort of 176 patients diagnosed with MDS ($n = 38$), AML ($n = 119$), or ALL ($n = 19$), who received an allo-HSCT at the Maastricht University Medical Center+ between January 2017 and October 2022, was selected for retrospective analysis of their pre-transplant STR profiles. This study used anonymised leftover human material and associated data, collected during routine clinical procedures. According to national regulations and institutional guidelines, no ethical approval or informed consent was required.

2.2 | HLA Typing

Low resolution HLA typing by the sequence specific oligonucleotide (SSO) technique was done using the LabType assay (Thermo Fisher Scientific) and the Luminex 3D instrument, following the manufacturer protocol. High resolution typing of patients and donors was performed by either Illumina sequencing, Sanger sequencing, or a combination of Nanopore (HLA class I) and Sanger sequencing (HLA class II). High resolution typing by Illumina sequencing was performed using the AllType FASTplex assay (Thermo Fisher Scientific) with a Miseq instrument, following the manufacturer protocol. High resolution typing by group-specific hemizygous Sanger sequencing was described previously [21], whereas high resolution typing by long-read Nanopore sequencing for HLA class I genes was in accordance with Matern et al. [22].

2.3 | STR Analysis

STR analysis was done according to the method of the Eurochimerism Consortium [23]. Twelve of the thirteen EuroChimerism markers were used for STR analysis (the STR marker P450-CYP19 was not used). To identify which markers are informative, all 12 were tested using DNA isolated from a pre-transplant peripheral blood sample of both patient and donor. The PCR consisted of three multiplex reactions, containing primers for STR markers D2S1360, D7S1517, D8S1132, D9S1118, and D17S1290; D10S2325, D11S554, D12S391 and SE33; MYCL1, D12S1064 and D19S253. The selected markers were confirmed in the same pre-transplant samples using a singleplex reaction and were subsequently used to monitor post-transplantation chimerism.

The PCR reaction mix of 25 μL consisted of 10 ng DNA, 5 pmol of each primer (Sigma-Aldrich, St. Louis, USA), 0.5 U AmpiTaq DNA polymerase (Thermo Fisher Scientific), 2.5 μL PCR buffer (50 mM KCl, 10 mM Tris–HCl pH 8.3 (GeneAmp 10× PE

TABLE 1 | Patient information of the 10 cases described in this study.

Case	Gender	Age at Tx	Malignancy	Risk stratification	Genetic mutation	Karyotype	Transplant type	Disease status at Tx	Donor type
1	Male	50	AML	Intermediate risk	NPM1 and FLT3	Normal	Allo	PD	MUD
2	Female	46	AML	Intermediate risk	NPM1 and FLT3	Normal	Auto	CR1	—
3	Female	37	AML	Favourable risk	NPM1	Normal	Allo	CR1	MUD
4	Male	57	AML	Intermediate risk	NPM1	Normal	Auto + Allo	CR1 + PD	Related haplo
5	Male	63	Secondary AML from MPN	Adverse risk	ASXL1	t(8;21) (6/20)	Allo	PD	Related 12/12
6	Male	58	Secondary AML from MDS	Favourable risk	IDH2	Normal	Auto + Allo	CR1 + PD	Related haplo
7	Female	N/A	ALL	Standard risk	None	Normal	None (refractory)	N/A	N/A
8	Male	52	CMML	Intermediate risk	ASXL1	cnLOH7q	Allo	CR1	Related haplo
9	Female	62	AML	Adverse risk	ASXL1	Complex (47,XX,+mar [19]/46,XX [1])	Allo	PD	MUD
10	Male	66	Secondary AML from MDS	Adverse risk	RUNX1, EVI1 and ASXL1	Monosomy 7 (19/20)	Allo	CR1	MUD

Abbreviations: ALL, acute lymphocytic leukaemia; AML, acute myeloid leukaemia; CMML, chronic myelomonocytic leukaemia; CR1, complete remission; MDS, myelodysplastic syndrome; MPN, myeloproliferative neoplasms; MUD, matched unrelated donor; N/A, not assessed; PD, progressive disease.

PCR buffer II, Thermo Fisher Scientific), 0.1 mg/mL cresol red (Sigma-Aldrich, St. Louis, USA), 0.2 mM of each dNTP (GE Healthcare, Diegem, Belgium), 5% Glycerol (Alfa-Aesar, Karlsruhe, Germany) and 1.5 mM $MgCl_2$ (Thermo Fisher Scientific)). The PCR reaction was performed as follows: initial denaturation at 95°C for 11 min, followed by 28 cycles of 60 s at 94°C, 60 s at 59°C, 60 s at 72°C, and final elongation at 60°C for 60 min. After resuspension in highly deionised formamide (Thermo Fisher Scientific) and GeneScan 500 LIZ dye size standard (Thermo Fisher Scientific), the PCR fragments were analysed on the ABI3730 DNA analyser (Applied Biosystems, Foster City, USA) and the Genemapper software 6 (Thermo Fisher Scientific) was used to analyse the size and the heights (RFU, Relative fluorescent units) of the fragment peaks.

3 | Results

3.1 | HLA Typing

Three AML patients demonstrated genetic variation of HLA in the leukaemic cells present in peripheral blood, one with the loss of an entire HLA haplotype (Case 1), one with a somatic mutation in HLA-A resulting in an amino acid change (Case 2) and one with a somatic mutation in HLA-B leading to a premature stop codon (Case 3).

3.1.1 | Case 1

In this case, the loss of an entire HLA haplotype in the leukaemic cells of a patient with AML was identified during the routine HLA typing performed before allo-HSCT. HLA typing of the patient was performed by low-resolution SSO typing. For the verification typing, high resolution was obtained by long-read Nanopore sequencing for HLA class I genes (HLA-A, -B and -C) and Sanger sequence-based typing for HLA class II genes (HLA-DRB1, -DRB345, DQB1 and DPB1) [21, 22]. The HLA typing using DNA from a peripheral blood sample with 85% circulating blasts resulted in a homozygous low and high-resolution typing for all the HLA genes (Table 2). Because of the homozygous typing result and the high blast count, a buccal swab was requested for retyping. Retyping with DNA isolated from the buccal swab resulted in a heterozygous typing result for all the HLA genes except HLA-DPB1.

Extended typing of HLA-DQA1 and DPA1 was done for both samples by Illumina sequencing (AllType FASTplex). The homozygous HLA-DPA1*01:03 typing in the buccal swab sample was as expected in association with the homozygous HLA-DPB1*04:01 typing.

This case demonstrates the identification of a complete loss of an HLA haplotype in leukaemic cells isolated from the peripheral blood of an AML patient. Such loss could have significantly impacted the donor selection if HLA typing had not been conducted using DNA isolated from buccal swab material. In this case, if the loss of heterozygosity had gone undetected, a homozygous unrelated donor might have been incorrectly selected as a 10/10 match, when in fact, this would only have been a 5/10 match.

TABLE 2 | Patients HLA typing result of DNA isolated from peripheral blood and buccal swab sample (Case 1).

Sample type	Blast %	Seq. approach	HLA-A		HLA-B		HLA-C		HLA-DRB1		HLA-DRB345		HLA-DQA1		HLA-DQB1		HLA-DPA1		HLA-DPB1	
PB	85%	SSO	01	—	51	—	01	—	11	—	3*02	—	05	—	03	—	NT	NT	NT	NT
		Nanopore/SSBT	01:01	—	51:01	—	01:02	—	11:01	—	3* 02:02	—	NT	NT	03:01	—	NT	NT	04:01	—
		Illumina	01:01	—	51:01	—	01:02	—	11:01	—	3* 02:02	—	05:05	—	03:01	—	01:03	—	04:01	—
Buccal swab	N.A.	SSO	01	02	15	51	01	03	04	11	3*02	4*01	03	05	03	03	NT	NT	NT	NT
		Nanopore/SSBT	01:01	02:01	15:01	51:01	01:02	03:04	04:01	11:01	3* 02:02	4* 01:03	NT	NT	03:01	03:02	NT	NT	04:01	—
		Illumina	01:01	02:01	15:01	51:01	01:02	03:04	04:01	11:01	3* 02:02	4* 01:03	03:01	05:05	03:01	03:02	01:03	—	04:01	—

Abbreviations: NT, not typed; PB, peripheral blood; SSBT, sanger sequenced-based typing; SSO, sequence-specific oligonucleotide.

3.1.2 | Case 2

The second case concerns the occurrence of a somatic mutation in exon 4 of HLA-A in the malignant cells of an AML patient. In the work-up for a related allo-HSCT, routine HLA typing using Illumina sequencing was performed for the patient and two siblings. Using DNA from a peripheral blood sample with a blast percentage of 84% (determined 4 days after the sample for typing was collected) the patient was typed as HLA-A*02:01new, A*03:01:01, B*07:02:01, B*27:05:02, C*01:02:01, C*07:02:01, DRB1*01:01:01, DRB1*15:01:01, DRB5*01:01:01, DQA1*01:01:01, DQA1*01:02:01, DQB1*05:01:01G, DQB1*06:02:01, DPA1*01:03:01, DPB1*04:02:01, and DPB1*126:01:01. The new HLA-A*02:01 allele was identified by a non-synonymous substitution in exon 4 at position 782 (G782A) (Figure 1A), corresponding to an amino acid substitution from glycine (codon GGG) to glutamic acid (codon GAG) at amino acid position 237 (Gly237Glu). The G782A substitution was confirmed when the sample was retyped using a second allele-specific full-length Sanger-based sequencing approach.

Due to the high blast percentage, a buccal swab was requested. The HLA-A typing of the buccal swab sample showed a discrepancy with the HLA-A typing obtained from the peripheral blood sample. The buccal swab DNA had HLA-A*02:01:01, whereas the peripheral blood DNA had HLA-A*02:01new (Figure 1B), indicating that the new allele was the result of a somatic mutation in the leukaemic cells of the patient. Accordingly, the HLA typing obtained from the buccal swab sample was identical to that of one of her sisters.

The sequence analysis of HLA-A in the peripheral blood sample showed that the nucleotide distribution within the reads that were assigned to HLA-A*02:01:01 at position 782 was 79% A and 21% G (with an average background percentage of 1% at the heterozygous positions in the exon regions), compared to 0% A and 100% G in the buccal swab sample. This was in line with the blast percentage of 84% determined by morphology. The amino acid Gly237Glu substitution is positioned in the alpha-3 domain of the HLA-A molecule (Figure 1C), which is outside the peptide binding groove of the protein. It is therefore probably not directly affecting peptide presentation or protein expression but might interfere with CD8 binding.

3.1.3 | Case 3

In the third case, the occurrence of a somatic mutation in the malignant cells of an AML patient resulted in the loss of an HLA-B allele. At the time of diagnosis, no information was available about the circulating blasts in this patient. Routine HLA typing at high resolution was performed using allele-specific Sanger sequence-based typing on DNA obtained from a peripheral blood sample: HLA-A*02:01:01, A*11:01:01, B*X, B*44:02:01, C*03:04:01,

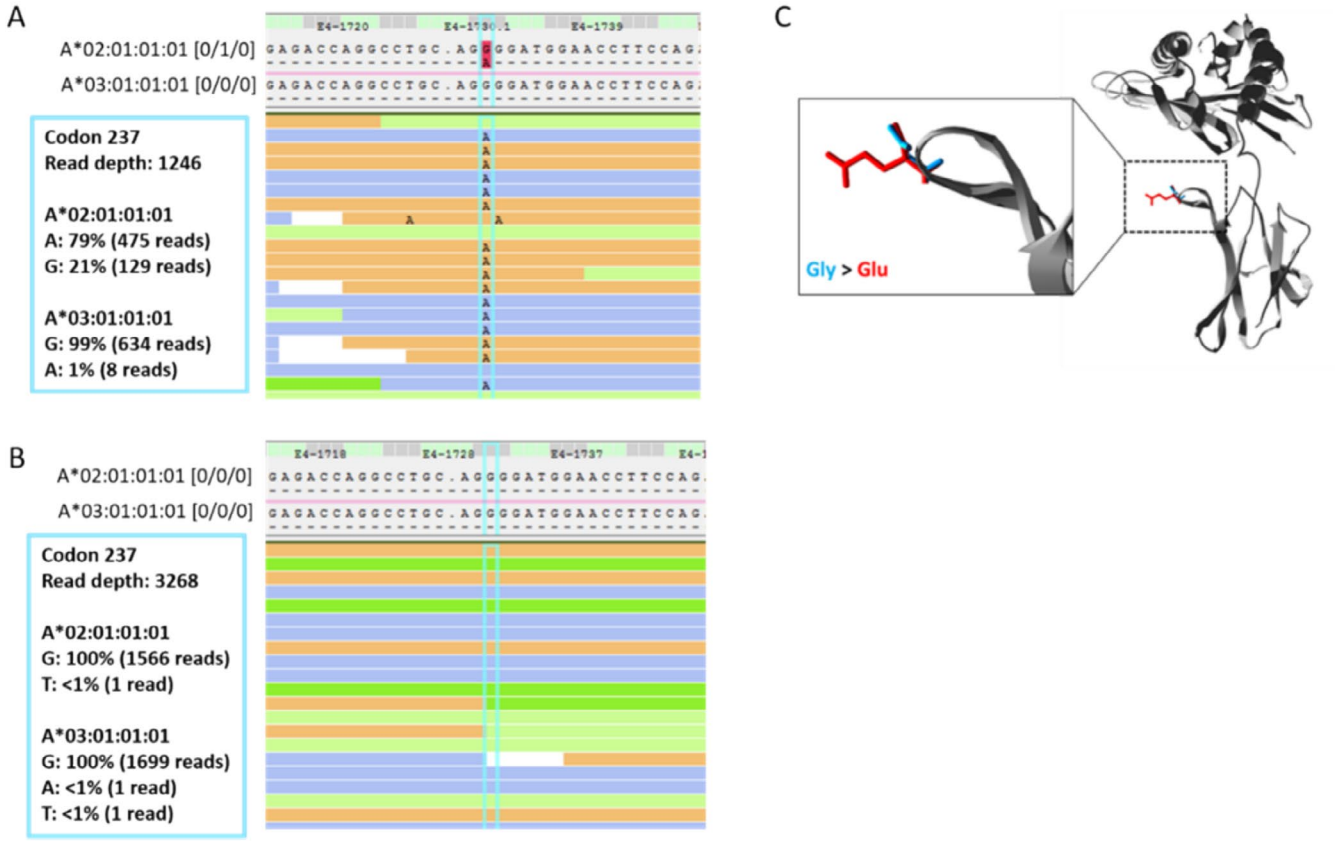

FIGURE 1 | Identification of a somatic mutation in HLA-A of a patient with AML (Case 2). Screenshot of the sequence analysis in Typestream, showing part of the Illumina reads for part of exon 4 of HLA-A*02:01:01:01; (A) The sequencing result of DNA isolated from a peripheral blood sample, with the G782A substitution, and (B) the sequencing result of DNA isolated from a buccal swab sample. (C) The effect of the Gly237Glu substitution on the protein structure of HLA-A*02:01:01:01.

C*07:02:01, DRB1*11:01:01, DRB1*13:02:01, DQB1*02:02:01, and DQB1*03:01. Allele assignment of HLA-B*X failed because the result did not match a known sequence in the IPD-IMGT/HLA database. Analysis of the electropherograms showed that the sequence was most similar to HLA-B*40:01, while missing 8 nucleotides in exon 2.

Since a complete HLA type was required for donor selection of this patient, retyping was performed using a sample that was collected 1 month after the initial sample, when the patient was in remission. Sequencing of this remission sample resulted in HLA-B typing of B*40:01:02 and B*44:02:01 typing, the HLA-A, -C, -DRB1, and DQB1 typing were identical to the typing obtained with the initial sample. This indicates that the deletion in HLA-B was possibly caused by the occurrence of a somatic mutation in the tumour cells present in peripheral blood at the time of diagnosis.

To investigate this somatic mutation in more detail, both samples were sequenced using Illumina sequencing, confirming the presence of a deletion of eight nucleotides (AGAGCAGG) in the exon 2 region of HLA-B*40:01 in the initial sample (Figure 2A). This deletion resulted in a frameshift and the formation of a premature stop codon at amino acid position 111. The deletion was not present in the HLA-B*40:01 sequence of the remission sample (Figure 2B). This premature stop codon is located 21 amino acids from the start of the alpha-2 domain of the HLA-B60 molecule, implicating that the molecule is not expressed on the surface of the malignant cells.

3.2 | Chimerism Testing

The following seven cases describe genetic alterations observed in the STRs of leukaemic cells, identified in peripheral blood samples during STR analysis prior to HSCT. Cases 4–7 show the presence of a third STR allele. In Cases 4, 5 and 6, it was detected at the time of diagnosis, and in Case 7, it was detected at diagnosis and retested at the time of relapse. Cases 8 and 9 show the partial gain or loss of an allele, and Case 10 describes the somatic alterations detected in three STRs within a single patient. Finally, the frequency of occurrence of these somatic changes within the tested STRs is described.

3.2.1 | Cases 4, 5 and 6

In the first three cases, the occurrence of a somatic insertion of one repeat in one of the STR markers was identified at the time of diagnosis in the cancer cells present in the peripheral blood.

In Case 4, three alleles were identified during the STR analysis of the D19S253 marker in a peripheral blood sample of an AML patient (Figure 3, Case 4A). The third allele appeared to be caused by a somatic insertion of one repeat, since only two alleles could be identified when the STR analysis was repeated with DNA isolated from a buccal swab sample of the patient (Figure 3, Case 4B). The percentage of cells in peripheral blood carrying the somatic insertion was calculated to be 62% at the moment of sample collection. Whereas the blast count determined in peripheral blood was 12% 1 day after the blood was collected for STR analysis.

In Case 5, STR analysis of a peripheral blood sample of a patient diagnosed with AML revealed three alleles in the MYCL marker (Figure 3, Case 5A). By testing a buccal swab sample, we showed that the allele with size 193 was caused by a somatic insertion of one repeat (Figure 3, Case 5B). The percentage of cells in peripheral blood carrying the somatic insertion was calculated as 38% at the moment of sample collection, compared to the blast count of 12% determined by morphology analysis.

In Case 6, three alleles were identified by analysis of the MYCL marker in the peripheral blood of a patient diagnosed with MDS (Figure 3, Case 6A), whereas only two alleles were present when a buccal swab sample of this patient was tested (Figure 3, Case 6B), indicating that the allele with size 206 might be the result of a somatic insertion of one repeat. It was calculated that 57% of the cells present in the peripheral blood at the moment of sample collection carried the somatic insertion. Again, this percentage was higher than the blast count of 27% determined by morphology.

3.2.2 | Case 7

The next case describes an ALL patient who was analysed using samples collected at the moment of diagnosis and at the moment of relapse.

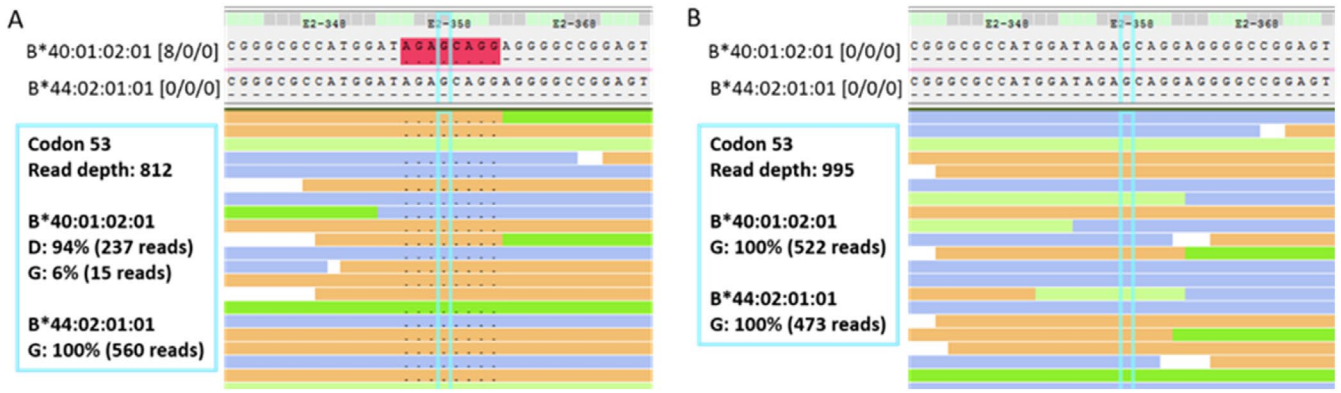

FIGURE 2 | Identification of a somatic deletion in HLA-B of a patient with AML (Case 3). Screenshot of the sequence analysis in Typestream, showing part of the Illumina reads covering part of exon 2 of HLA-B*40:01:02:01; The sequencing result of DNA isolated from a peripheral blood sample (A) at the time of diagnosis, showing the deletion of 8 nucleotides, and (B) at the time of remission.

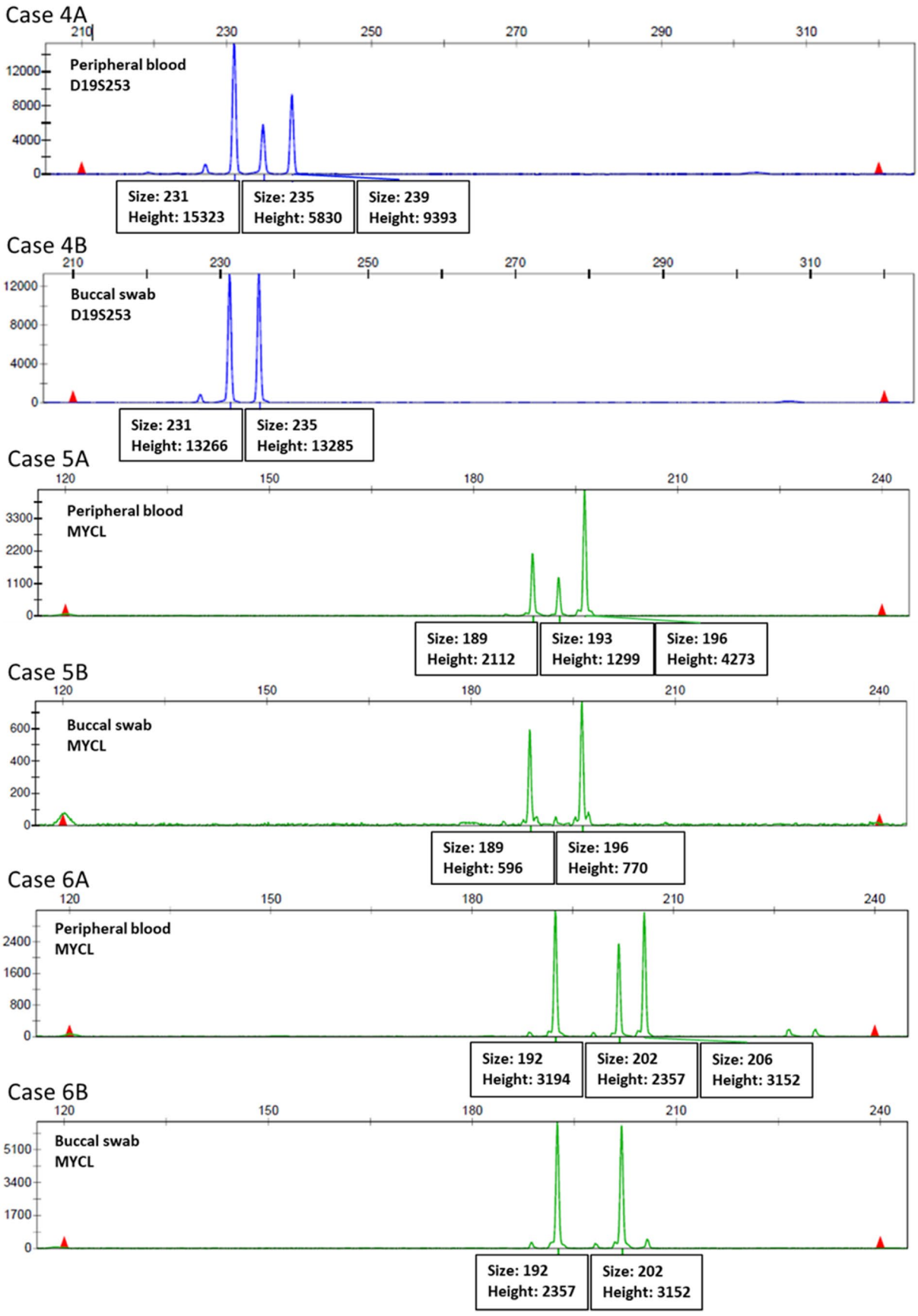

FIGURE 3 | Short tandem repeat profiles of indicated samples for marker D19S253 in an AML patient (Case 4A + B), for marker MYCL in an AML patient (Case 5A + B) and for marker MYCL in a MDS patient (Case 6A + B).

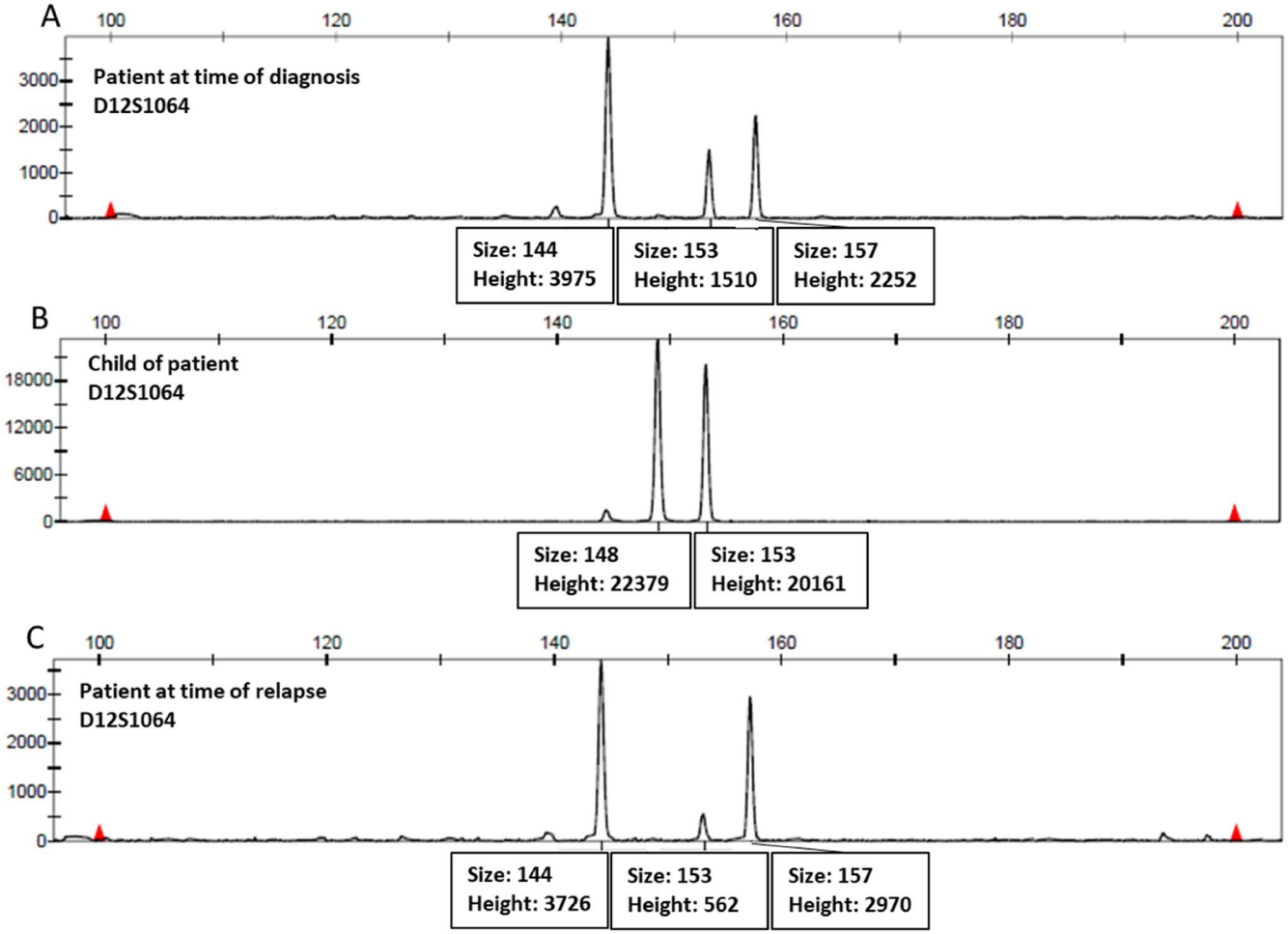

FIGURE 4 | Short tandem repeat profiles of indicated samples for marker D12S1064 in an ALL patient (Case 7) and the child of the patient.

In the peripheral blood sample of this ALL patient at the time of diagnosis, three alleles were detected with the D12S1064 marker (Figure 4A), suggesting a somatic mutation in the leukaemic cells. This finding was supported by the high blast percentage of 52% in the peripheral blood of this patient 1 day before the sample for STR analysis was collected.

Unfortunately, there was no buccal swab sample of the patient available to repeat the analysis. Since the patient was prepared for a related haplo-identical HSCT as treatment, DNA of two children was available. The STR analysis indicated that the presence of the third allele with size 157 in the patient's sample might be caused by an insertion of one repeat (Figure 4B).

Interestingly, similar results were found when the STR analysis was repeated with a sample that was collected when the patient was in relapse, approximately 2 years after the first sample and without transplantation treatment during this period. Again, three alleles with identical sizes as found in the sample collected at diagnosis were identified (Figure 4C), suggesting that the same leukaemic clone as at the moment of diagnosis might be causing the disease recurrence. No blast percentage was determined in the same blood sample, but the blast percentage was 70% in a sample collected 3 days after the collection of the sample for STR analysis. The higher blast percentage compared to the first sample fits with the STR analysis results, as reflected by the decreased height of the peak with allele size of 153 and the increased height of the peak with allele size 157.

3.2.3 | Cases 8 and 9

The next two cases show that somatic loss of a chromosome can lead to the partial or total loss of an allele and thereby interfere with the STR analysis.

Case 8 describes the occurrence of somatic variation in the STR region of a patient diagnosed with CMML. The STR analysis of the D7S1517 marker showed two alleles, but with an unexpectedly large height difference between the two peaks (4716 vs. 785 RFU) (Figure 5A). This height difference disappeared when the analysis was repeated with DNA isolated from a buccal swab sample of the patient (1333 vs. 1449 RFU) (Figure 5B). This result suggests the (partial) loss of chromosome 7 in part of the cells, where the D7S1517 STR marker is located, as the reason for the lower presence of an allele with size 204. Indeed, the patient's karyotype showed copy-neutral loss of heterozygosity of chromosome 7q. The percentage of blasts was not known at the time the peripheral blood sample was collected.

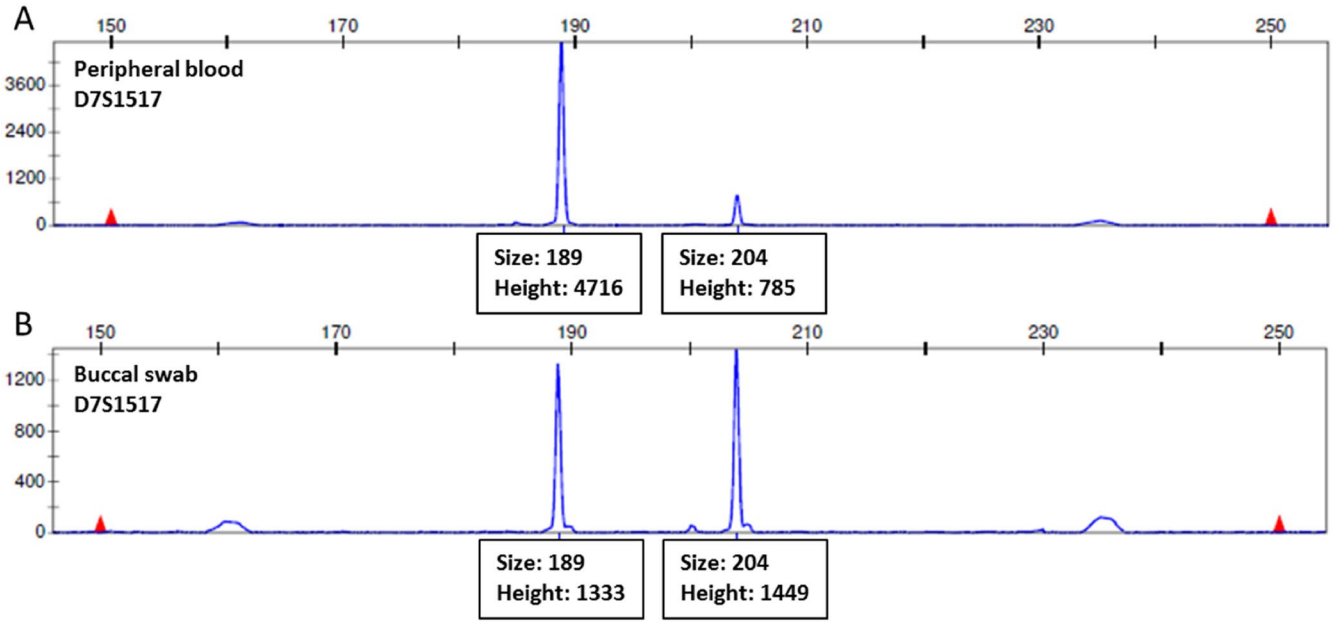

FIGURE 5 | Short tandem repeat profiles of indicated samples for marker D7S1517 in a CMML patient (Case 8).

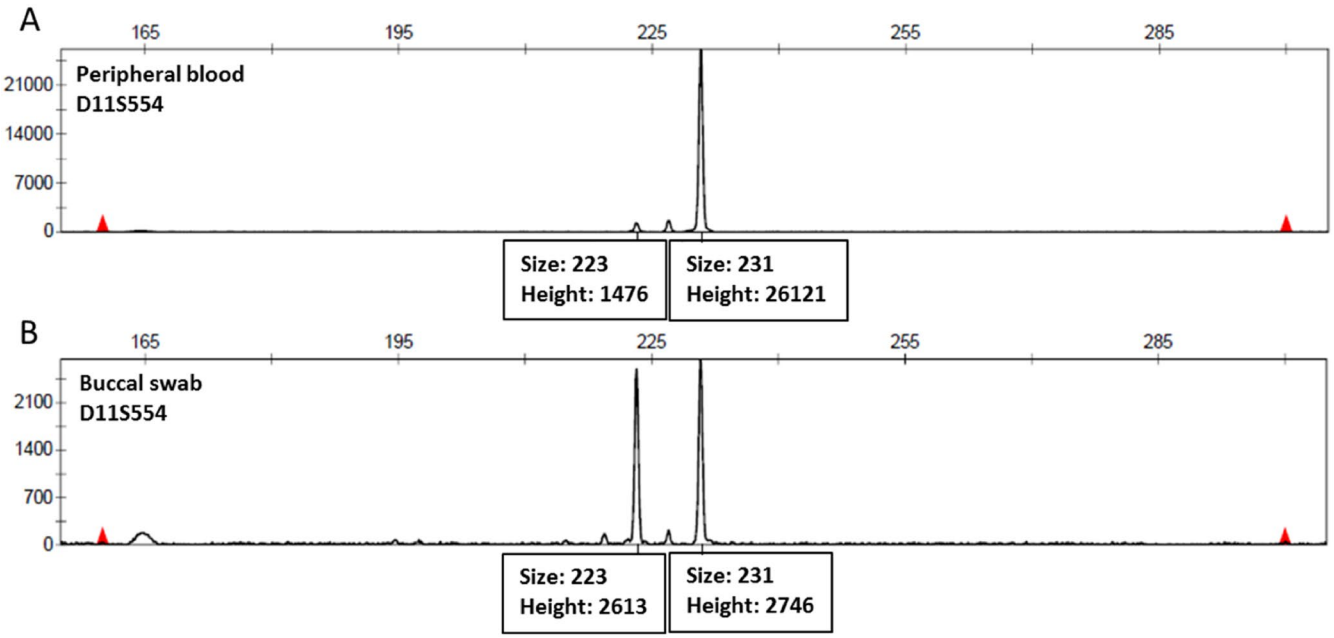

FIGURE 6 | Short tandem repeat profiles of indicated samples for marker D11S554 in an AML patient (Case 9).

Case 9 shows the occurrence of somatic alteration of chromosome 11 in an AML patient. The STR analysis of the D11S554 marker showed the presence of one allele with size 231; the two other peaks were initially interpreted as 'stutter' artefact peaks (Figure 6A). However, since the blast percentage of the peripheral blood sample was 89%, a buccal swab sample was requested, and the analysis was repeated, revealing the presence of two alleles (Figure 6B). These results indicate the presence of tumour cells in the peripheral blood carrying a somatic (partial) deletion of chromosome 11, which could not be directly explained by the patient's karyotype, although it contained an unidentified marker chromosome. The percentage of cells that carried a deletion of chromosome 11 was calculated to be 94%. If the analysis had not been repeated using a buccal swab sample, there was a risk that the marker could have been selected for chimerism analysis, since the peak with size 231 was informative compared to donor peaks of marker D11S554. This case shows that in a very high percentage of cells with somatic chromosomal alterations, the STR analysis can be influenced in such a way that an incorrect selection can lead to an erroneous chimerism determination.

3.2.4 | Case 10

In an AML patient, the STR analysis of a peripheral blood sample collected at the time of diagnosis identified the occurrence of somatic alterations in 3 different STR markers (D11S554,

D12S391 and D7S1517). For D11S554, three alleles were present in the peripheral blood sample (Figure 7A), compared to only two alleles in a buccal swab sample (Figure 7B), suggesting that a somatic insertion of one repeat is present in approximately 50% of the peripheral blood cells. Similarly, STR analysis of D12S391 revealed the presence of three alleles in the peripheral blood sample (Figure 7C), whereas only two alleles were present in the buccal swab sample (Figure 7D), indicating a somatic insertion of one repeat in 44% of the leukaemic cells.

For D7S1517, the STR analysis of the peripheral blood sample resulted in two peaks (Figure 7E), with an unexpectedly large height difference (1050 vs. 2972 RFU). In comparison, in the buccal swab sample, both alleles were present in equal amounts (Figure 7F), suggesting the presence of either a somatic insertion of one repeat or a deletion of one STR allele. Karyotyping of this patient indeed revealed monosomy 7, indicating that the large difference in the presence of both alleles is due to the loss of chromosome 7. The percentage of cells in the peripheral blood with monosomy 7 was based on the STR analysis calculated to be 65%. Interestingly, the blast percentage was only 5% in a peripheral blood sample collected 1 day before the sample for STR analysis was collected.

3.3 | Follow-Up Post-Transplant

As part of the follow-up for the 10 cases described, we repeated the analysis using post-transplant samples, if available, to determine whether the somatic alteration reappeared after transplantation, particularly in cases of relapse (Table 3). We used patient samples collected for chimerism analysis and selected either the sample taken at the time of relapse or the one with the highest patient chimerism percentage. Two of the 10 patients (Cases 2 and 7) did not undergo allogeneic transplantation, and of the eight patients who did, three experienced relapse (Cases 1, 5 and 9).

In the leukaemic blast cells of the Case 1 patient, the loss of an entire HLA haplotype was detected at diagnosis. High-resolution HLA typing of the sample at relapse revealed the same homozygous result as observed prior to transplantation, suggesting the re-emergence of the same leukaemic clone. In the post-transplant peripheral blood and bone marrow samples at relapse in the Case 5 patient, the same somatic insertion of one repeat in the MYCL marker was identified, indicating that the somatic alteration had reappeared with the relapse. The Case 9 patient also relapsed after transplantation, but the loss of one of the D11S554 STR alleles was not detectable in the post-transplant sample. For the five patients who did not relapse after transplantation, the somatic alteration was, as expected, not detectable in the post-transplant samples.

3.4 | Occurrence of Somatic STR Variation

To investigate the occurrence of somatic variation within the STR regions at the moment of diagnosis of patients, we analysed 176 patients with MDS, AML, or ALL who underwent an allo-HSCT in the MUMC+ between January 2017 and October 2022. Three patients whose cases are described in this study also met these criteria (Cases 5, 6 and 10); the other cases in this study included patients who have not had a transplant within this period. The pre-transplant STR multiplex results of the 176 patients were used to check for the presence of a third allele, indicating a somatic insertion or deletion of a repeat in an STR, or an unexpected difference between the prevalence of the two alleles, indicating the loss of one allele. In the case of a potential somatic variant, the STR analysis was repeated using a singleplex reaction of the same marker to confirm the finding.

By analysing the 176 multiplex results, we found in 6% ($n = 11$) of the patients a somatic variant in one ($n = 9$), two ($n = 1$) or three ($n = 1$) of the STRs, of which 8 resulted in the presence of a third allele and 6 in the partial gain or loss of an allele (Table 4). The somatic variation occurred in 9 of the 12 STRs tested and more than once in 3 of the STRs. The STRs with a somatic variant were located on chromosomes 1, 2, 6, 7, 8, 11, 12 and 17, but were not found on the STRs located on chromosomes 9, 10 and 19.

Four of the six cases with partial gain or loss of an allele could be explained by the patient's karyotype at diagnosis; loss of a D7S1517 and D12S1064 allele was correlated with monosomy 7 and monosomy 12, respectively, whereas gain of a D8S1132 allele correlated with trisomy of chromosome 8. Because the results have been obtained retrospectively, most tests could not be repeated with DNA isolated from a buccal swab sample due to sample availability, and therefore it could not always be determined whether it was gain or loss of one or more repeats.

4 | Discussion

Peripheral blood samples from patients with haematological malignancies often contain blast cells carrying somatic alterations within regions of HLA or STR loci. Identifying these somatic alterations prior to HSCT is critical, as demonstrated by the patient-based cases of this study, since they can result in HLA mistyping and affect the results of STR analysis essential for chimerism monitoring.

In the first case, the presence of leukaemic blasts in the peripheral blood led to an incorrect homozygous typing result, underscoring the difficulty in distinguishing somatic alterations from normal HLA homozygosity. By mixing two samples that were completely homozygous for HLA at different ratios, we determined the threshold for detecting heterozygosity with the AllType FASTplex assay to be 15% (data not shown). This highlights that incorrect homozygosity does not require a sample with 100% tumour cells. Moreover, this threshold is assay- and kit-specific, necessitating determination for each different assay [9, 11].

Detection of somatic mutations could also result in the identification of false new alleles, as exemplified in Cases 2 and 3, making it challenging to distinguish them from genuine novel variants. Additionally, somatic mutations might be missed if the somatic variant results in a typing of an already known HLA allele. In the IPD-IMGT/HLA database, there are several alleles known that differ only by one nucleotide, for example, HLA-B*44:02:01:01 and HLA-B*44:05:01:01 [24]. We must consider whether all new HLA alleles added to the IPD-IMGT/HLA database are germ-line variants or if some were discovered as variants caused by somatic mutations. Moreover, somatic mutations

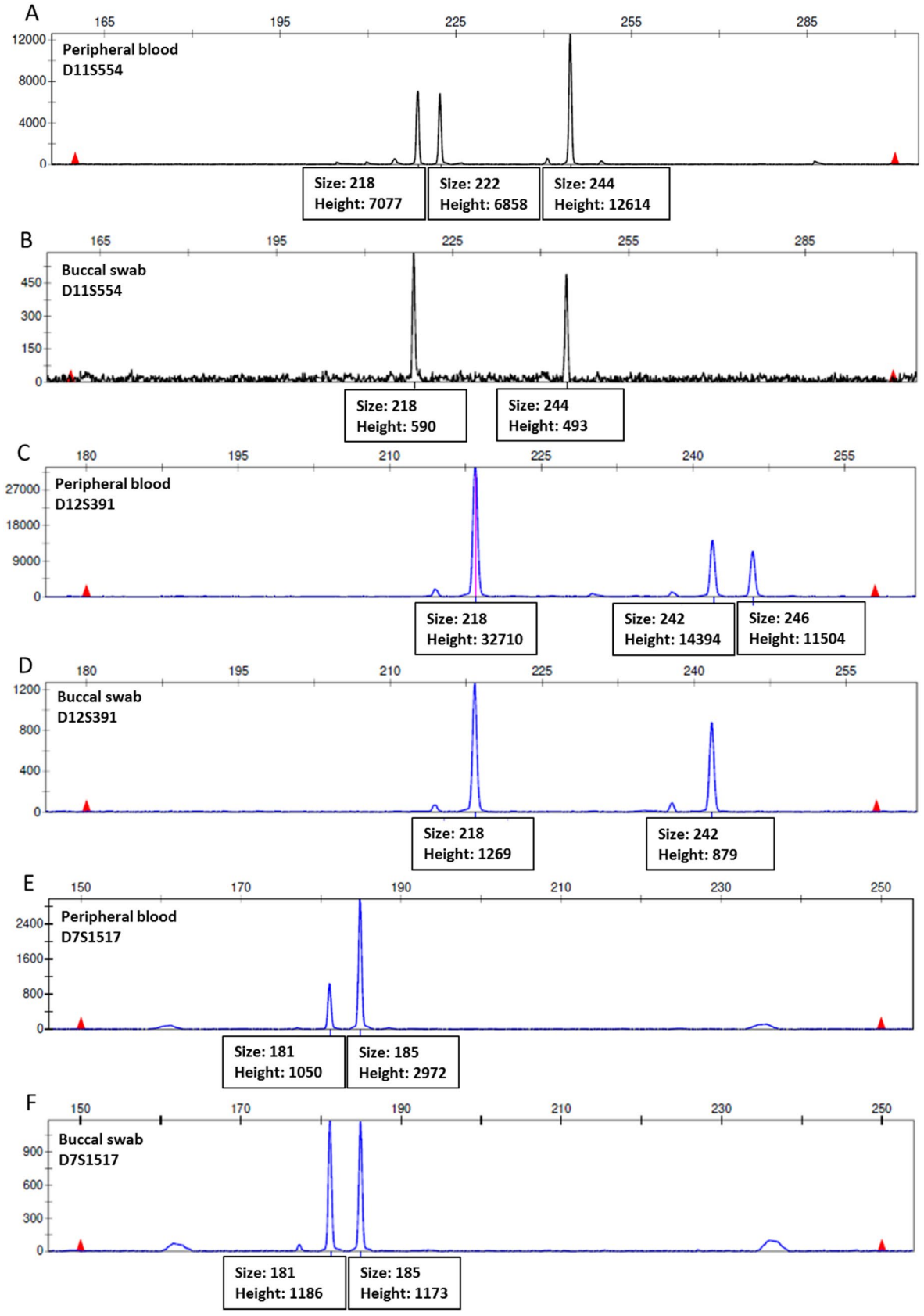

FIGURE 7 | Short tandem repeat profiles of indicated samples for markers D11S554 (A + B), D12S391 (C + D) and D7S1517 (E + F) in an AML patient (Case 10). Analysis of these markers shows the effect of somatic variation through the presence of a third allele or the partial loss of an allele.

TABLE 3 | Post-transplant analysis of somatic alterations in the 10 patient cases described in this study.

	Post Tx sample					Closest follow-up	
Case	Received	Days after Tx	Patient chimerism	Relapse status	Pre Tx somatic alteration detectable	Patient status	Days after Tx
1	PB	81	78%	+	Yes	Dead	< 100
2	None	N/A	N/A	N/A	NA	Alive, MDS	> 1000
3	PB	80	< 1%	−	No	Alive	> 1000
4	PB	31	< 1%	−	No	Dead	< 100
5	PB + BM	65	18% + 48%	+	Yes	Dead	< 100
6	PB	376	< 1%	−	No	Alive	500–1000
7	None	N/A	N/A	N/A	NA	Dead	NA
8	PB	158	< 1%	−	No	Dead	100–500
9	PB	63	36%	+	No	Alive[a]	500–1000
10	PB	292	< 1%	−	No	Alive	500–1000

Abbreviations: BM, bone marrow; MDS, myelodysplastic syndrome; N/A, not assessed; PB, peripheral blood.
[a]Patient was in remission after donor lymphocyte infusion treatment.

TABLE 4 | Cases of somatic variation detected by a specific STR marker in 176 patients who underwent an allo-HSCT between 2017 and 2022.

STR marker	Location	Presence of third allele	Partial gain/ loss of allele
MYCL1	1p34	2× (Case 5 + Case 6)	—
D2S1360	2p24	—	1×
SE33	6q15	1×	—
D7S1517	7q31	1×	2× (1× Case 10)
D8S1132	8q23	—	1×
D9S1118	9p21	—	—
D10S2325	10p13	—	—
D11S554	11p11	3× (1× Case 10)	—
D12S1064	12q21	—	1×
D12S391	12p13	1× (Case 10)	—
D17S1290	17q21	—	1×
D19S253	19p13	—	—

within the primer binding region might result in an allele drop-out, resulting in an erroneous homozygous HLA typing result that could be misinterpreted as correct.

Failure to correctly identify HLA loss or a somatic HLA mutation in the patient sample can influence donor selection based on HLA matching. A donor might be selected who actually does mismatch the patient's non-tumour cells, or a compatible donor might be excluded based on a mismatch with the patient's tumour cells. Therefore, HLA typing of the recipient must be repeated before transplantation for verification using a different sample. This can help to identify mix-ups and errors in HLA typing. In addition, it may detect somatic mutations in the cells of one of the samples. However, if both typing samples are obtained from peripheral blood, it is still possible that both contain cells with the somatic mutation, leading to identical but incorrect HLA typing. For patients with haematological malignancies where tumour cells can occur in the peripheral blood (e.g., MDS, AML and ALL), it is recommended to perform HLA typing on DNA isolated from an alternative source, such as a buccal swab sample, at least once, or, alternatively, to confirm the patient's HLA typing by comparing it with the HLA typing of family members, if possible.

This study also describes somatic alterations in the STR loci of seven different patients, detected during the STR analysis on pre-transplant peripheral blood samples. Many of these somatic alterations were observed when STR analysis was repeated on buccal swab material. Notably, in the majority of cases, the blast count determined by morphology was lower than the calculated percentage of tumour cells with a somatic STR variation. A possible explanation for this discrepancy is that the blast count determination and STR analysis were not performed on the same patient sample; the samples were collected at different times, and in some cases, on different days. For patients with progressive disease, these timing differences could lead to major variations in the percentages of blast cells in the peripheral blood, potentially contributing to the observed lower blast counts based on morphology compared to the chimerism by STR performed at a later time point.

This study presents findings from a total of 18 instances of somatic alterations in STRs, demonstrating gains or losses of STR alleles across 8 different chromosomes, many of which correlate with the respective patient's karyotype. Haematological malignancies, such as AML, often exhibit diverse chromosomal

abnormalities that can potentially influence STR analysis. For instance, deletions involving chromosomes 5 and 7 are frequently observed in AML. Monosomy 5 or deletion of 5q is estimated to occur in 10%–15% of all AML cases, while chromosome 7-related abnormalities, including monosomy 7 or del(7q), are observed in about 5% of adult AML cases [25–27]. Although abnormalities in other chromosomes are less frequent, various complex abnormalities, such as trisomy of chromosome 8, can also occur in AML [28]. Given the high prevalence of chromosomal abnormalities like those involving chromosomes 5 or 7, caution may be warranted when considering including STRs located in these regions in chimerism panels.

A limitation of our study is that the 6% of somatic alterations in the tested STRs is an estimation, as not all analyses could be replicated using DNA isolated from buccal swab samples due to limited sample availability. Nevertheless, this frequency aligns with a previous study which reported a similar percentage of 7% after investigating somatic STR variations in 72 AML and myelofibrosis patients following HSCT [16]. These data indicate that somatic alterations are common in patients with acute leukaemia, making it critical to identify these somatic alterations prior to transplantation, as they can lead to incorrect calculations if these regions are chosen for chimerism testing following HSCT. To minimise the risk of inaccurate results, it is recommended to establish informative systems for chimerism analysis using DNA isolated from buccal swab material and considering cytogenetic data when selecting STR markers. If it is not possible to obtain an alternative sample, such as buccal swab material, it could be beneficial to use additional STR loci for chimerism testing along with determining the coefficient of variation, if feasible, to increase the likelihood of detecting any discrepancies in the results [29]. Alternatively, other chimerism detection methods, such as SNP-based assays or digital PCR, could be used. These approaches rely on multiple genetic markers across various chromosomes, primarily SNPs, which are less susceptible to somatic alterations than STRs. By analysing multiple markers, the likelihood of false positives or outliers is reduced, minimising the potential impact of somatic alterations on chimerism detection.

While buccal swabs are a convenient source of DNA, they may occasionally be contaminated by malignant cells in leukaemia patients, especially if small amounts of blood or oral abnormalities, such as gingival hyperplasia, are present. To minimise the risk of misleading results, we recommend visually inspecting buccal swabs for blood contamination and considering alternative sampling methods, like hair follicles or skin biopsies, in high-risk cases.

Among the 10 cases described in our study, three of the eight patients who underwent allo-HSCT experienced relapse. In two cases, the somatic alteration identified at diagnosis was also detected at relapse, indicating clonal persistence and suggesting that relapse originated from the same leukaemic clone. Conversely, in the third case, the somatic alteration present at diagnosis was absent at relapse, suggesting that the disease recurred from a genetically distinct clone. These observations are consistent with the established literature on clonal evolution in AML and other myeloid malignancies, which highlights that relapse can arise from residual leukaemic clones present at diagnosis or from new clones selected under therapeutic pressure [30–33].

This study highlights the importance of detecting somatic alterations before HSCT, as they can interfere with HLA and STR analysis, leading to mistyping or incorrect chimerism detection. Accurate sample sourcing, such as using buccal swabs, is recommended when tumour cells are present in peripheral blood. Considering the patient's karyotype when selecting STR markers can improve diagnostic accuracy. Establishing clear guidelines for these diagnostics will enhance the reliability of pre-transplant assessments.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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