CASE REPORT

False homozygous HLA genotyping results due to copy number neutral loss of heterozygosity in acquired aplastic anaemia

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SUMMARY

The aim of this case report is to draw attention on possible false human leucocyte antigen (HLA) genotyping in acquired aplastic anaemia prior to allogeneic haematopoietic stem cell transplantation. In acquired aplastic anaemia loss of heterozygosity (LOH) of chromosome 6p is known to occur in around 12%. We report false HLA genotyping results due to LOH and a coinciding steep rise in neutrophils following filgrastim stimulation in a patient with very severe aplastic anaemia. At diagnosis we obtained heterozygous results on peripheral blood. Failing to reach a partial response at 6 months with immune-suppressive therapy we repeated HLA genotyping, obtaining homozygous results. Repeated testing confirmed loss of HLA genotype heterozygosity. HLA genotyping on cells obtained by a buccal swab confirmed the previous HLA heterozygosity. A second course of filgrastim at the time of homozygous HLA genotyping resulted in a steep rise in neutrophils. Stopping filgrastim resulted in an equally steep drop.

BACKGROUND

The aim of this case report is to draw attention on possible false human leucocyte antigen (HLA) genotyping in acquired aplastic anaemia (aAA)

prior to allogeneic haematopoietic stem cell transplantation.

aAA is a bone marrow failure syndrome resulting in reduced peripheral blood counts. Autoreactive cytotoxic T lymphocytes (CTLs) are thought to be responsible for immune destruction of haematopoietic stem cells.¹ Clonal evolution is a well-described complication in aAA and arises in >70% of patients.² This is manifested by the detection of a paroxysmal nocturnal haemoglobinuria (PNH) clone in up to half of the patients¹ and malignant evolution to myelodysplastic syndrome (MDS) or acute myeloid leukaemia in 15% of patients.³ Two papers were published describing the frequency and clinical impact of copy number neutral loss of heterozygosity (CN-LOH) of chromosome 6p. Frequency was 13% in a Japanese cohort⁴ and 11.3% in a Caucasian cohort.⁵ In aAA, CN-LOH clones tend to be stable in time without the development of MDS-associated cytogenetic abnormalities⁵ in the Caucasian cohort. Chromosome 6p carries the HLA locus. Loss or downregulation in HLA class I antigens is a way to escape CTL surveillance.⁶ This escape mechanism is identified in a variety of malignant conditions.^{7,8} Hypothesis was made of the same escape mechanism from autoreactive CTLs in aAA.⁹ It is described in aAA even without previous therapy.⁵ In severe aplastic anaemia, a treatment algorithm can be followed in which the availability of a matched sibling donor plays a central role.¹⁰

Table 1 Results of HLA genotyping at the time of diagnosis on peripheral blood and at follow-up after 7 months on peripheral blood and cells obtained from buccal swab

Specimen	Blood	Blood	Buccal swab
Date tested	Diagnosis*	7 months†	7 months†
HLA-A1	*24	*24:02	*24:02
HLA-A2	*33	— (lost)	*33:01
HLA-B1	*07	*07:02	*07:02
HLA-B2	*14	— (lost)	*14:02
HLA-C1	*07	*07:02	*07:02
HLA-C2	*08	— (lost)	*08:02
HLA-DR1	*15	*15:01	*15:01
HLA-DR2	*13	— (lost)	*13:03
HLA-DQB1	*06	*06:02	*06:02
HLA-DQB2	*03	— (lost)	*03:01

Heterozygous results are found at diagnosis on peripheral blood whereas genotyping on peripheral blood at 7 months shows homozygous results (loss of HLA-A*33, HLA-B*14, HLA-C*08, HLA-DR*13, HLA-DQB*03).

*Low resolution: Luminex Multiplex typing, Immucor Lifecodes, Nijlen, Belgium.

†High resolution: HLA sequencing, Olerup, Wien, Austria. HLA, human leucocyte antigen.

CASE PRESENTATION

A 57-year-old woman of Caucasian origin without any medical history was admitted to the haematology ward for a work up of pancytopenia. She was referred by her general practitioner who performed

Figure 1 Chest X-ray at diagnosis of invasive fungal infection (left panel) and after 2 months of treatment (right panel). At diagnosis a pulmonary infiltrate is seen in the right lower lobe whereas at follow-up the volume of this lesion has decreased significantly.

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Figure 2 Bone marrow biopsy, H&E stain, $\times 40$. No signs of dysplasia.

a complete blood count because of general fatigue that had worsened since several weeks. After bone marrow biopsy, we withheld diagnosis of very severe aAA (bone marrow cellularity $< 25\%$ with three cytopenias: haemoglobin 4.9 g/dL, thrombocytes $2000/\text{mm}^3$, neutrophils $98/\text{mm}^3$). Low resolution HLA typing by PCR sequence-specific oligonucleotide (SSO) using the Labtype HLA-SSO kits (One Lambda, Uden, the Netherlands) demonstrated a heterozygous genotype for all class I and II loci (table 1). DNA was extracted using the MagNa Pure Compact System instrument and the Nucleic Acid isolation kit 1 (Roche Diagnostics, Mannheim, Germany). Karyotype (G banding analysis+fluorescence in situ hybridisation) was normal. No matched sibling was found. Immunosuppressive therapy with ciclosporin, antithymocyte globulin and methylprednisolone was initiated. During aplasia, 5 weeks following admission, pain developed in the right hemithorax. Chest X-ray demonstrated a large infiltrate in the middle of the right hemithorax (figure 1). Diagnosis of a probable invasive fungal infection by EORTC (European Organisation for Research and Treatment of Cancer) criteria was made. First-line therapy with voriconazol was started but was complicated by toxic hepatitis. We switched to liposomal amphotericin B and added filgrastim $300 \mu\text{g}$ per day. After 77 days she left our department. For another month we treated her with liposomal amphotericin B

and filgrastim three times per week resulting in an almost complete resolution of the pulmonary infiltrate (figure 1). Neutrophils around $500/\text{mm}^3$ still remained until further tapering and eventually stopping filgrastim, which caused them to drop to around $250/\text{mm}^3$. She never became platelet transfusion independent. Seven months after diagnosis she developed spondylodiscitis of C4-C5. Intravenous therapy with vancomycin was started based on different cultures of peripheral blood showing *Staphylococcus aureus* and *S. hominis*. Neutrophils at that time were $213/\text{mm}^3$. We restarted filgrastim, resulting in a steep rise in neutrophils to just under $3500/\text{mm}^3$. After 20 days we stopped filgrastim resulting in an equally steep drop in neutrophils to $500/\text{mm}^3$. Not reaching a partial response (defined by transfusion independence¹¹) with immunosuppressive therapy and in the absence of an HLA compatible sibling, we considered allogeneic transplantation with an unrelated donor. A second high-resolution HLA typing by Sanger sequence using the sequence-based typing (SBT) resolver kits (Olerup, Wien, Austria) demonstrated homozygous results for all of the HLA-A, HLA-B, HLA-C, HLA-DRB1 and HLA-DQB1 loci (table 1). A new blood sample confirmed the homozygous HLA genotype. HLA genotyping on buccal swab cells by SBT confirmed the first HLA heterozygous typing results (table 1). New bone marrow biopsy did not show any signs of dysplasia (figure 2) but cytogenetic analyses (G banding analysis) showed monosomy 7 while chromosome 6 appeared unimpaired (figure 3). During the course of disease, she received multiple transfusions with packed cells and thrombocytes. No granulocyte infusions were given since she responded well to antibiotic and antifungal medications and neutrophil numbers could be increased with granulocyte-colony stimulating factor.

DISCUSSION

This patient, suffering from very severe aAA and developed copy number neutral loss of HLA heterozygosity. The hypothesis of escape from CTL surveillance by loss of heterozygosity (LOH) is confirmed by the response to filgrastim in our patient. Only the second course of filgrastim resulted in a steep increase

Figure 3 G-banding: karyogram of bone-marrow cells using Giemsa staining. Loss of one chromosome 7 (monosomy 7) is seen. Other chromosomes including chromosome 6 are unimpaired.

in neutrophil count and an equally steep decrease when stopping filgrastim. HLA genotyping was heterozygous when the first course of filgrastim was started but was homozygous at the start of the second filgrastim course. This suggests that the HLA homozygous clone is more responsive to filgrastim or more successful in escaping immune recognition by autologous CTL when stimulated by filgrastim. To the best of our knowledge, no other cases have been published that showed this remarkable rise and fall of neutrophils following filgrastim stimulation in patients with CN-LOH.

In a Caucasian cohort CN-LOH clones tend to stabilise in time without development of MDS-associated cytogenetic abnormalities.⁵ This is in contradiction with our patient who developed monosomy 7, which is known as a high-risk MDS-associated abnormality. However, no signs of dysplasia were found on bone marrow biopsy (figure 2).

In haematological malignancies attention has been drawn before to possible false HLA genotyping prior to transplantation.¹² In our patient cytogenetic analysis failed to identify this problem. Chromosome 6 appeared unimpaired when homozygous HLA genotyping was found. Sanger sequencing is currently the golden standard method in typing HLA at high-resolution level, due to its high accuracy and simple chemistry. This sequencing method is suitable to analyse all the polymorphisms of the targeted exons (particularly exons 2–4 for class I loci and exon 2 for class II loci).

The biggest drawback of this method is the inability to phase the heterozygous sequences (combinations of many pairs of HLA alleles) and therefore in many cases, HLA genotyping ‘ambiguity’ remains an issue. HLA typing by Sanger sequencing requires several rounds of additional testing to provide high-

resolution level by which the turnaround time of a sample increases.

The Sanger method includes poor quality in the first bases of the sequence due to primer binding and deteriorating quality of sequencing traces after 700–900 bases and therefore it can sequence limited read length. Finally, Sanger sequencing has a lower sensitivity in detecting minor alleles than PCR-SSP.¹³

In our patient HLA-B*14 and HLA-C*08 were lost. Both were described in the Caucasian cohort with aplastic anaemia to be more affected.⁵ Owing to prior heterozygous HLA genotyping in our patient, immediate attention was drawn to the new homozygous results and LOH was suspected and confirmed. Homozygous HLA genotype results prior to stem cell transplantation must always raise suspicion in order to avoid haematopoietic transplantation with a mismatched donor.

Contributors The first author was responsible for literature research and writing the manuscript. ADB supervised the clinical follow-up of the patient. SV is responsible for all HLA testing. CD noticed the discrepancy between the new and earlier HLA results and motivated to write this manuscript. All co-authors were equally involved in critically revising and editing the manuscript. The final version was approved by all authors.

Competing interests None declared.

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Learning points

- ▶ Homozygous human leucocyte antigen (HLA) genotyping results prior to stem cell transplantation must always raise suspicion of loss of heterozygosity.
- ▶ Loss of heterozygosity of HLA genotype as a result of alterations in chromosome 6p is a frequent clonal evolution in acquired aplastic anaemia.
- ▶ This homozygous clone most likely escapes cytotoxic T lymphocytes resulting in a possible better response to filgrastim.
- ▶ The haematological patients' HLA typing must always be repeated on a newly collected sample or with another type of sample such as a buccal swab and if possible by a different HLA typing technique.
- ▶ If possible, homozygosity of HLA has to be proven by family studies of the patient. HLA typing must be carried out on available members of the immediate family (parents or siblings).

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