Human leucocyte antigen matching and donor selection for sibling bone marrow transplantation in India

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Fifty one patients undergoing treatment for various diseases and disorders (leukaemia of different type, thallasaemia and anaemia) at Kothari Medical Centre, Kolkata and awaiting bone-marrow transplant, and their siblings were studied for their Human Leucocytes Antigen (HLA) compatibility. Forty of these patients had siblings available as probable donors. In eleven cases, planned pregnancy and testing the chorionic villi sample (CVS) was adopted. The study revealed that the probability of finding a full house match was greater with larger sib-ship size tested: 63% with three available sibling donors, 43% with two and 36% with only one sibling available. CVS donors also showed full house match in 36% of the cases. Five of the families were studied for HLA –A*, B* compatibility as well. When the DRB1* locus was a probable homozygous, HLA A* and B* typing revealed their incompatibility.

HAEMATOPOIETIC stem cells transplant from healthy matched donors is potentially life saving for patients with fatal inherited or acquired diseases including haematological malignancies[1,2]. Haematopoietic stem cells have the capacity for continuous renewal and for generating committed progenitors capable of replenishing matured functional blood cells and immune system. Thus engraftment of stem cells from a normal donor corrects genetic abnormalities and malignancies, rescues patients from toxic drugs and rehabilitates them. The successful engraftment largely depends on the degree of Human Leucocytes Antigen (HLA) matching between the donor and the recipient, and with incompatible grafts, severe graft versus host disease, an immunological reaction will ensue, killing the patient. Paradoxically, the stem cell graft containing immunologically matured T-cells with minor incompatibility can also provide a potent graft versus leukaemia reaction, effectively reducing the risk of post-transplant relapse[3].

A major constraint of this valuable therapeutic transplant is the need of a full house, i.e. 100% HLA matched donor. It is now well established that the success of a transplant is dependent on the degree of HLA matching in live related donor transplant, and also HLA mismatching in live related and unrelated donors obtained from donor registry, i.e. the population donor (e.g. www.anthonynolan.org.uk). The influence of HLA matching is the immunological basis of graft rejection and this is better understood with bone marrow transplantation than with any other organ transplantation. The probability of severe clinical grades III–IV acute GVHD (Graft Versus Host Diseases) was 48% for DRB1 matched and 70% for DRB1 mis-matched unrelated donor transplant[1]. Hence, the HLA matching is absolutely mandatory for bone-marrow transplant (BMT) for various immunological reasons: after all the patient's family is interested in the success of BMT and the rehabilitation of the patient.

In India, the field of bone marrow transplantation is still in its infancy. Lack of education and public awareness, lack of trained manpower and dedicated hospitals, high hospital infection rates and lack of matching facility are the factors jeopardizing the growth of this field. This elegant form of therapy that will be highly valuable to patients and families concerned needs to be implemented and offered to common citizens of this country. We present our observations on matching live related sibling donor and chorionic villus sample (CVS) for bone marrow transplantation, in a patient cohort attending the Haematological Unit, Kothari Medical Centre, Kolkata.

Fifty one patients attending the Medical Centre, (Table 1) from 14 July 1999 to 20 May 2003 opted for bone marrow transplantation. The line of treatment and options available were discussed with the family members by the attending physicians and families were motivated to donate bone marrow/stems cells for transplantation. Eleven families without any sibling other than a mismatch were counselled and underwent a planned pregnancy. These CVS samples were also HLA tissue typed to identify the potential donor. The number of CVS cases was 11 and the number of adult sibling donors was 40.

Table 2 presents the distribution of diseases in the patients: Chronic myeloid leukaemia (13/51) and thalassaemia (20/51) were more common than other diseases. Peripheral blood from patients and potential donors were obtained in EDTA vacutainers and shifted to Madurai by courier service.

Table 1. Statewide distribution of patients opting for BMT at Kothari Medical Centre

State/country	No. of patients	Percentage
West Bengal	38	74.51
Rajasthan	3	5.88
Gujarat	2	3.92
Punjab	1	1.96
Uttar Pradesh	2	3.92
Bihar	1	1.96
Orissa	1	1.96
Pakistan	1	1.96
Nepal	1	1.96
Bhutan	1	1.96
Total	51	100

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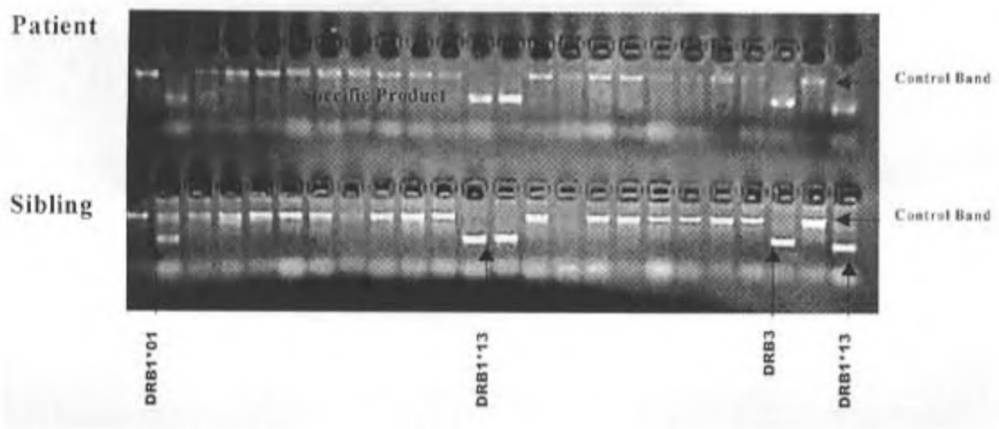

Figure 1. Photograph of an agarose gel electrophorogram of a patient and his sibling donor: Twenty-four DRB1* group specific primer sets were used to amplify the alleles in 24 PCR reactions, the products electrophoresed in different lanes and documented. Note the presence of control bands in all the lanes and specific bands in the same specific lanes, in both the patient and sibling. Inference: Donor and recipient are 100% (full house) matched for DRB1*.

Table 2. Distribution of diseases among BMT patients attending Kothari Medical Centre, BMT facility and opting for BMT

Disease	Patients
Acute lymphoblastic leukaemia	6
Aplastic anaemia	6
Chronic myeloid leukaemia	13
Thalassaemias	19
Acute myeloid leukaemia	5
Glanzmann's thrombaspenia	1
Hypoplastic anaemia	1
Total	51

DNA was extracted from peripheral blood using salting out procedure[4]. HLA DRB1* SSP typing was performed following the methods described earlier[5]. Sequence-specific primers were synthesized following the sequences published earlier[5] and presented in Table 3. Polymerase chain reaction (PCR) temperature profiles recommended by Olerup and Zetterquist[5] and as modified by the protocol of Dynal and One lambda were followed respectively as per manufacturer's instructions. In short, a two-stage temperature cycle was adopted using an MJ Thermal cycler (PTC-1196, MJ Research). The PCR thermal cycling consisted of denaturation at 94°C for 2 min, 10 cycles of 2-step PCR with 94°C for 10 s and 65°C for 60 s, followed by 20 cycles of 94°C for 10 s, 61°C for 50 s and 72°C for 30 s. The PCR products were electrophoresed in 2% agarose gel at 100 V for 20 min and documented in a Kodak digital imaging System under UV illumination (VI549393-Kodak digital science, USA) (Figure 1). HLA A*, B* generic typing was performed by SSP method, employing commercial trays as per the manufacturers' protocol (SSP1 A, SSP1 B, One lambda, USA).

An in-house reference panel of eleven DNA samples was sequenced to know the exact alleles: SBT typing method[6] using DRB1* primer mix was employed to sequence both DRB1* alleles of a sample simultaneously, in an ABI PRISM 310 automated sequencer (31000220, Perkin-Elmer, USA). This reference panel was used to quality control the DRB1* typing. All the indicated alleles were identified at the generic level by the in-house primer sets.

In addition, PCR-SSP DRB1* typing trays from One Lambda (SSP2LB) and Dynal (550.01) were also employed. Selected reference samples and samples with ambiguous results in in-house reagents, were tested on commercial trays. The in-house trays gave concordant results and the missing reaction was mostly due to PCR failures. Hence, either one or more than one tray, was used for typing each family. Whenever an ambiguity was identified or allele was missing, the test was repeated and/or a new batch of primers or commercial trays were used for PCR.

Figure 1 shows the results of a patient and his sibling donor. A control band of 750 bp was identified in all the wells. This indicated that the PCR has worked well in all the reactions. The alleles were identified by the presence of a specific band of indicated size in respective wells (Table 3; Figure 1). Note the identical pattern of bands in both the patient and the donor in agarose gel electrophorogram (Figure 1). This was a case of full house match, both patient (BMT recipient) and donor sharing the same alleles, DRB1*01, DRB1*13 and DRB3.

Table 4 presents the distribution of full house, 50% (haplo-identical) and no match cases, among 3, 2 or 1 donor sib-ship families. There were eight families with three sibling donors and five of them had full house matching (63%). Seven patients had two sib-ship donors available and only 3 of them were found to have full house match (43%). In 25 families with one sibling donor available, 9 of them

Table 3. Sequences of primers synthesized and used in SSP typing to identify DRB1* alleles at generic level (ref. 5; DYNAL)

PCR no.	DRB1* allele identified	Forward primers		Reverse primers		Product size (bp)
		Name	5′ Sequence 3′	Name	5′ Sequence 3′	
1	*01	5′01	TTGTGGCAGCTTAACTTGAAT	3′047	CTGCACTGTGAAGCTCTCAC	255
				3′048	CTGCACTGTGAAGCTCTCCA	255
2	*0301-02	5′03	TACTTCCATAACCAGGGAGGAGA	3′03	TGCAGTAGTTGTCCACCCG	151
3	*04	5′04	GTTTCTTGGAGCAGGTTAAACA	3′047	CTGCACTGTGAAGCTCTCAC	260
				3′048	CTGCACTGTGAAGCTCTCAC	260
4	*07	5′07	CCTGTGGCAGGGTAAGTATA	3′079	CCCGTAGTTGTGTCTGCACAC	232
5	*08	5′08	AGTACTCTACGGGTGAGTGTT	3′045	TGTTCCAGTACTCGGCGCT	161
				3′18	GCTGTTCCAGTACTCGGCAT	163
6	*09	5′09	GTTTCTTGAAGCAGGATAAGTTT	3′079	CCCGTAGTTGTGTCTGCACAC	236
7	*10	5′10	CGGTTGCTGGAAAGACGCG	3′047	CTGCACTGTGAAGCTCTCAC	204
8	*11	5′05	GTTTCTTGGAGTACTCTACGTC	3′06	CTGGCTGTTCCAGTACTCCT	176
9	*12	5′08	AGTACTCTACGGGTGAGTGTT	3′08	CACTGTGAAGCTCTCCACAG	248
10	*1301–1302	5′03	TACTTCCATAACCAGGAGGAGA	3′10	CCCGCTCGTCTTCCAGGAT	130
11	*1303–04	5′05	GTTTCTTGGAGTACTCTACGTC	3′045	TGTTCCAGTACTCGGCGCT	171
12	*1401, 1404–05	5′05	GTTTCTTGGAGTACTCTACGTC	3′11	TCTGCAATAGGTGTCCACCT	224
13	*1405	5′08	AGTACTCTACGGGTGAGTGTT			215
14	*1305, 1402–03	5′03	TACTTCCATAACCAGGAGGAGA	3′12	TCCACCGCGGCCCGCC	140
15	*15	5′02	TCCTGTGGCAGCCTAAGAG	3′01	CCGCGCCTGCTCCAGGAT	197
16	*16	5′02	TCCTGTGGCAGCCTAAGAG	3′02	AGGTGTCCACCGCGGCG	213
17	*0301	5′06	GACGGAGCGGGTGCGGTA	3′048	CTGCACTGTGAAGCTCTCCA	217
18	*0302, 1302, 1305, 1402–03	5′03	TACTTCCATAACCAGGAGGAGA	3′047	CTGCACTGTGAAGCTCTCAC	189
19	DRB3	5′52.1	TTTCTTGGAGCTGCGTAAGTC	3′13	CTGTTCCAGGACTCGGCGA	171
20	DRB3	5′52.2	GTTTCTTGGAGCTGCTTAAGTC	3′14	GCTGTTCCAGTACTCGGCAT	173
21	DRB4	5′53	GAGCGAGTGTGGAACCTGA	3′048	CTGCACTGTGAAGCTCTCAC	213
22	DRB5	5′51	GTTTCTTGCAGCAGGATAAGTA	3′01	CCGCGCCTGCTCCAGGAT	200
				3′16	CCGCGGCGCGCCTGTCT	207
23[Ψ]	GH	GH-FOR	GCCTTCCCAACCATTCCCTTA	GH-REV	TCACGGATTTCTGTTGTGTTTC	429

Ψ, Dynal Technical Handbook.

Table 4. Distribution of full house (100%), haplo-identical (50%) and nil (0%) matches among families with different sib-ship donors size

Sib-ship donor available	No of families studied	Number of families with a matched donor (%)		
		Full house	Haplo-identical	No match
3	8	5 (63%)	6 (75)	2 (25)
2	7	3 (43)	5 (100)	1 (14)
1	25	9 (36)	13 (52)	3 (12)
1 (CVS)	11	4 (36)	5 (45)	2 (18)

had full house match (i.e. 36%). It is logical that the probability of finding a full house matched donor was greater with larger sib-ships available for donor selection.

Families with thalassaemic patients not having any sibling or no full house matched sibling donor for bone marrow donation, were advised to undergo planned pregnancy. Eleven thalassaemic patient's families returned for CVS sample testing. Four of them were found to be full house match (36%) (Table 4). This was similar to the full house match obtained in families with one sibling donor.

Five families with full house match or one DRB1* blank match requested further investigations for HLA A*, B* match, to confirm the haplotypes. The results revealed that HLA A*, B* were fully matched in three families with both the DRB1* allele identified and full house match (Table 5). In the two families with only one DRB1* allele identified (putative homozygous), it turned out to be a HLA A*, B* one haplotype mismatch between the putative donor and patient (Table 5).

The approach that can be adopted in India for an efficient and cost effective BMT matching has been demonstrated. One of the teething problems in this field of transplant therapy in India is the non-availability of good and quality HLA matching facility. With the advent of DNA and PCR technology, preparation of quality reagents (primers), unlike serological method, quality control and shipping of the reagents/templates either as blood or DNA, is much more easier. Serological methods of yester-years heavily

Table 5. HLA DRB1*, A* and B* compatibility in selected BMT families

F. No	ID. No	Sex	Age (years)	Relationship	Disease	DRB1*	DRB1*	DRB3	DRB4	A*	A*	B*	B*	Percentage matching
1	8849	F	5	Patient	Thallasaemia	7	12	3	4	11	24	15	35	100
1	8850			CVS	Healthy donor	7	12	3	4	11	24	15	35	
2	9054	M	44	Patient	CML	1	13	3	–	3	31	35	51	100
2	9055	M	47	S1	Healthy donor	1	13	3	–	3	31	35	51	
2	9085	M	34	S3	Healthy donor	1	13	3	–	3	31	35	51	
3	9954	M	12	Patient	Aplastic anaemia	7	9	–	4	1	11	52	57	100
3	9955	F	4	S1	Healthy donor	7	9	–	4	1	11	52	57	
4	8918	M	7	Patient	Thallasaemia	7	Blank	–	4	33	24	44	Blank	50
4	8919		0	S1	Healthy donor	7	Blank	–	4	33	1	44	55	
5	8922	M	3	Patient	Thallasaemia	4	12	3	4	2	11	52	13	50
5	8923			Cord blood	Healthy donor	4	Blank	3	4	2	24	52	15	

When a DRB1* allele is a blank (family no 4 and 5), there was one allele mismatch in HLA-A* and B* loci, i.e. only a 50% match between the probable donor and patient; DRB3 and DRB4 loci were supertypic to various lineages of DRB1* locus; 100%, full house match; 50%, Haploidentical.

depended on live lymphocytes, quality antisera, 'alive' complement and hence was highly demanding. With the DNA technology, many of these problems and quality control issues were easily solved. If research institutions and universities in every city having a good PCR laboratory infrastructure extend BMT HLA matching services, we can improve the quality of our health care delivery system: such establishments in private and corporate sector hospitals turn out to be costlier for common man.

Screening of family members, particularly siblings, is the best method of selecting a donor for bone marrow transplantation. This is the most cost effective method for the family concerned compared to donor obtained from population donor registry, such as Anthony Nolan (www.anthonynolan.org.uk). India is in an advantageous position in this direction: due to strong family structure and availability of more than one sibling in many families finding a full house match becomes easier. Even with only one available sibling donor or CVS as potential donor, the chances of obtaining a full house match was 36%, as identified in the present study. More the number of siblings available for testing as donors, greater the chance of finding a donor: the chances increased to 63% in three sibling potential donor family (Table 4).

The present study has also revealed that screening for HLA DRB1* polymorphism of the patient and the potential sibling donor is an efficient and cost effective way of identifying a probable donor. As described in Table 5, when both the DRB1* alleles of the patient and the potential donor have been identified, it may be a case of full house match across the Major Histocompatibility Complex (MHC) region including HLA A* and B*. Nonetheless, when there was a blank in DRB1* locus (i.e. an allele could not be assigned, presumably homozygous at the generic level), the likelihood that HLA A* and B* alleles may be different is greater. The present study confirmed this: when two such probable DRB1* homozygous families were tested for HLA A*, B* they turned out to be one allele mismatch in these loci (Family Nos 4 and 5), and hence not a suitable bone marrow donor. Thus matching for HLA DRB1* in the first instance and further investigating for HLA A*, B* is a cost effective and pragmatic approach of donor selection in India.

The study has also revealed the utility of planned pregnancy in donor selection. By this method of testing CVS samples, 36% of the patients could obtain a potential donor. Though this is a cumbersome way of counselling, planning pregnancy and waiting, in diseases like thalassaemia where there is no immediate, imminent danger for life, this would prove to be the best method for these families. When the results were informed to the family of the patients and they were counselled, though it was a non-thalassaemic CVS sample and a HLA mismatch, none of the families resorted to abortion: the families were keen on giving birth to a non-thallassaemic child, it seems.

Finally, an HLA tissue matching laboratory needs to take all the precautions to maintain quality: In our experience, high stringency of reagents and techniques, validation of commercially available anti-HLA sera and DNA typing kits are a prerequisite of a tissue matching laboratory[7,8]: false negatives and false positives and wrong assignment of alleles affect the outcome of graft survival. In Western countries bone marrow donor programmes, low resolution or intermediate resolution DNA testing is often used to screen a large pool of donors. If a HLA-matched donor is identified, allele level typing is then performed. These marrow donor programmes require patients to have high resolution matched DRB1* and intermediate level typing for HLA – A* and B*.

High resolution testing is less critical when the potential donor is a sibling with the same biological parents as the patient, as has been confirmed in the present study. Whenever both HLA DRB1* alleles were identified and

the donor and the recipient were fully matched, the HLA A*, B* typing further confirmed them to be both the haplotypes matched (full house match) (Table 5). Thus under Indian conditions, matching for HLA DRB1*, HLA A* and B* and perfect match of 6 out of 6 allele is sufficient enough in the case of sibling donation[9]. (www.bmtinfonet.org.newsletters.issue53.perfectdonor.usa.html).

A tissue-matching laboratory needs to establish inhouse reference standards, run reference standards with every new batch of reagents, confirm in repeat typings and confirm with commercially available reference reagents as and when required. In the absence of clinical laboratories coming forth to establish this kind of state-of-the-art facilities due to various reasons such as the high cost involvement, lack of understanding, lack of technical manpower, etc. a partnership between research institution/universities and the local hospitals aspiring to offer this kind of state-of-the-art therapy is the need of the hour and will be highly beneficial to the families concerned.

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Green hairy root cultures of *Solanum khasianum* Clarke – a new route to *in vitro* solasodine production

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Solasodine is recognized as a potential alternative to diosgonin. Both share the characteristic that they can be converted to 16-dehydro-pregnenolone acetate; the first step in steroid synthesis. Effort has been ongoing in the production of solasodine using cell and lately hairy root cultures of various *Solanum* species. As seen in our pilot experiments, some of the hairy root lines of *Solanum khasianum* Clarke show enhancement of solasodine production compared to non-transformed roots. Light and temperature as physical factors of growth are found to have an important role in controlling greening, growth and secondary product formation in these hairy root cultures. This is similar to the greening observed in tubers of *Solanum tuberosum* (potato), leading to stimulation of solanine production. Enrichment of the nutrient medium with CO_2 further increases growth and secondary metabolite production in the hairy root cultures of *S. khasianum*. Thus by manipulating environmental and nutritional conditions, solasodine production can be maximized in hairy root cultures of *S. khasianum*.

SEVERAL morphological changes are observed in hairy root cultures when exposed to light, viz. change in biomass as a direct effect of light[1], and greening of root clones[2]. The physiological effects of light are thought to be due to the total amount of energy (intensity of light) that has been incident on an organ or tissue rather than the daylength of phytochrome-controlled process[3]. Root formation in green protocorms of *Cymbidium* is reported to occur only at high intensity of light[3], while light stimulates greening in *Solanum tuberosum* tubers at low temperature[4]. Ramaswamy *et al.*[5] suggested that total glycoalkaloids in potato are formed within the chloroplast. Therefore, as a result of greening, the total glycoalkaloid can be increased in potato. Chloroplast may become starch-containing amyloplasts when tubers are grown in the dark. Given appropriate stimuli of light, amyloplasts may be converted to chloroplasts and vice versa[3]. Hence it is thought interesting to stimulate greening in the hairy root cultures of *Solanum khasianum* using light and to study its effect on solasodine production. Green roots developed under light conditions could be used for increased secondary metabolite synthesis[6–11]. In plants, sugars favour the expression of enzymes in connection with biosynthesis and storage of reserves, while repressing the expression of enzymes involved in

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