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Research article

HLA-DR, -DP, -DQ expression status of parathyroid tissue as a potential parathyroid donor selection criteria and review of literature

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ABSTRACT

Background: Basic and clinical studies about parathyroid allotransplantation have to be utilized with more definitive criteria for longer graft survival. Several reports demonstrated different isolation and cultivation methods for parathyroid cells to minimize their immunogenicity. In this study, we aim to compare and evaluate the clinical characteristics and the status of HLA class II expression changes in parathyroid tissue.

Methods: A total of 22 parathyroid hyperplasia tissue donors was included in this study. Clinical characteristics were evaluated and compared with the HLA-DR, -DP, -DQ mRNA, and protein expression levels which were determined by qRT-PCR and Western blot.

Results: We have compared the clinical characteristics (age, dialysis duration, frequency, recurrency of hyperparathyroidism and, calcimimetic usage) and HLA class II expression. HLA class II mRNA and protein levels showed varied expression patterns between tissues. Only, the HLA-DP has high mRNA expression levels without affecting the protein level when compared with the ages of the tissue donors. In addition, the HLA-DR, HLA-DP, and HLA-DQα1 protein expression levels showed a permanent and varied expression rate between tissues.

Conclusion: Expression of HLA class II molecules in parathyroid cells appears to constitute a decisive factor. Despite the lack of clinical outcomes, present data proposes new insight with a detailed understanding of parathyroid immunogenicity. In the future, randomized controlled clinical trials are needed for the accurate assessment of the effect of the varied HLA class II expression profiles in parathyroid tissue.

1. Introduction

The parathyroid tissue is composed of chief, oxyphil, and water clear cells and the percentage of cell type in each parathyroid gland among individuals is highly heterogeneous. A most recent study from Ritter et al. reported a different cell type which is referred to as "transitional oxyphil cells" [1]. As is known, the parathyroid oxyphil cells are markedly increased in chronic kidney diseases. These cells include more eosinophil than oxyphil cells but are closer in size to the chief cells [1,2]. Therefore, a limited group of researchers reported that oxyphil cells are derived from chief cells, and "transitional oxyphil cells" increase a heterogeneous cell size distribution in parathyroid tissue [1,3-5].

To our best knowledge, in the last 40 years, several reports defined different isolation and cultivation methods for parathyroid cells to minimize parathyroid cell immunogenicity, particularly for transplantation. Most *in vitro* attempts aimed to decrease human leukocyte antigen (HLA) expression by cultivation or cryopreservation [6-10]. With the studies reported so far, parathyroid cells were prepared in different culture media formulations before transplantation including Dulbecco's modified version (DMEM) [11], Chang Medium® C [7], AmnioMAX™-II Complete Medium [10], and McCoy's 5A Medium [12]. Despite using different additives, the immunogenicity of the parathyroid cells was evaluated by Nawrot et al., and in their study, the cells in Chang C medium were treated with IFN for six weeks. This method provided transplantable cells

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with > 85% viability [7]. However, the transplantation outcome was reported as 55.1% even with a delicate and expensive cultivation process to minimize the HLA expression levels [6].

By this study, we suggest a revised approach that the importance of transplantable parathyroid donor tissue selection. This should include different features while comparing HLA expression status. Herein, we have divided the parathyroid tissue donors based on their varied clinical characteristics such as dialysis duration, dialysis frequency, age at donation, recurrent donation and, calcimimetic usage for proper parathyroid tissue selection. Then we compared the mRNA and the protein expression levels of HLA class II including HLA-DR, -DP, and -DQ.

2. Materials and methods

2.1. Donors recruitment

Twenty-two patients with parathyroid hyperplasia due to chronic renal failure (mean age 40.4 years, range 23–64 years) who were referred from the nephrology outpatient clinic to the general surgery department for surgical approach were enrolled into the study. Donors were scheduled for parathyroidectomy and those who had hepatitis B, hepatitis C, or human immunodeficiency virus (HIV), or who had their parathyroid tissue damaged during surgery were excluded from the study. Tissue was evaluated morphologically and then half of each of the resected glands was delivered to the pathology department for diagnosis. The remaining parts of the glands were transported by using an ice-cold Parathyroid Transportation Solution ® (BAVU Solution®) [13] to the parathyroid cell culture laboratory unit and prepared for isolation.

2.2. Cell preparation

The cell isolation process generated by the method described previously [13,14]. Cell viability was assessed using a Muse Cell Analyzer (Merck Millipore, Darmstadt, Germany) with a Muse Count & Viability Assay Kit (Merck Millipore, Darmstadt, Germany). After viability assessment, $1x10^6$ cells were cultivated for 24 h, then supernatant samples were collected and kept at −80 °C. Then parathormone (PTH) levels from supernatant samples were determined with a commercially available human EIA kit (RayBiotech Inc., Norcross, GA, USA). The remaining cells were immediately prepared for the total protein and total RNA isolation process.

2.3. Preparation of total protein extracts and Western blot

A 200 μl complete RIPA lysis buffer (containing 2 μl phenylmethylsulfonyl fluoride, 2 μl sodium orthovanadate solution, and 2 μl protease inhibitor cocktail) (Santa Cruz, TX, USA) was used. The protein lysates were frozen overnight at −80 °C. Then extract was centrifuged for 15 min at 13,000 *g*, and then the supernatant was collected. The total protein concentrations were measured by using a Qubit Protein Assay Kit (Thermo Fisher Scientific MA, USA). A 50 μg sample of total protein extract was separated on 4–20% SDS-polyacrylamide gradient gels (Bio-Rad, CA, USA) and transferred to 0.2 μm PVDF membranes (Bio-Rad, CA, USA) in a 1X transfer buffer that contains 20% MeOH at 2.5 V for 10 min with the Trans-Blot Turbo Transfer System (Bio-Rad, CA, USA). The membranes were blocked overnight at + 4 °C with TBS-T (Tris Buffered Saline with 0.02% Tween-20) containing 5% nonfat milk. The primary antibody for HLA-DR was mouse monoclonal Anti-HLA-DR Antibody (1:1000 dilution, clone TAL 1B5, Thermo Fisher Scientific MA, USA), HLA-DP was rabbit polyclonal Anti-HLA-DP Antibody (1:500 dilution, Abcam, Cambridge, UK), HLA-DQA1 antibody was rabbit monoclonal Anti-HLA-DQA1 Antibody (1:5000 dilution, clone EPR7300, Merck Millipore, Darmstadt, Germany), HLA-DQA2 antibody was rabbit polyclonal Anti-HLA-DQA2 Antibody (1μg/mL, Abcam, Cambridge, UK) β-actin was rabbit polyclonal (1:1000 dilution, Cell Signaling, MA, USA) and anti-rabbit and/or anti-mouse IgG, HRP-linked Antibody was used as the secondary antibody when necessary (1:3000 dilution, Cell Signaling, MA, USA). The membranes were washed 5 times (5x5 minute), each between antibody incubations with TBST. The blots were developed using the ECL detection kit (Bio-Rad, CA, USA).

2.4. Total RNA Isolation and qRT-PCR

Extraction of total RNA was performed with a High Pure RNA Isolation Kit (Roche, Mannheim, Germany) according to the manufacturer's recommendations. Quantitative analysis of RNA was conducted by measuring optical density at 260 nm and 280 nm using Multiskan GO (Thermo Fisher Scientific, MA, USA). The isolated RNA was used as a template for cDNA synthesis in reverse transcription reactions using a First Strand cDNA Synthesis Kit (Invitrogen, CA, USA) followed by the manufacturer's instructions. Then, qRT-PCR was performed using SensiFast™ SYBR No-ROX reagents (Bioline Reagent, Ltd., London, UK) and the Bio-Rad CFX96 instrument (Biorad, CA, USA). Primer sequences for HLA-DR; F:ACCTGGTTGCTACTGGTTCG, R: ATCAATGCTGGGACTTCAGG, HLA-DP; F:GACCCTGTGAAATACTGTAAAGGTG, R:GGGCAGCTGGAGTTCAGA and HLA-DQ, F: TTGTGTTCCCACCCTTGG, R: AAGGGCAGACGGTATCCAT. qRT-PCR was conducted using the following program: 95 °C for 2 min followed by 40 cycles of (denaturing at 95 °C for 5 s, annealing at 61 °C (60.3 °C HLA-DR) / 60 °C (HLA-DP) / 60.3 °C (HLA-DQ) for 15 s, and extension at 72 °C for 10 s), with a final extension step at 72 °C for 2 min. The ratios of *HLA-DR*, *HLA-DP*, *HLA-DQ* relative mRNA expression levels were calculated using the $2^{-\Delta\Delta}$ Ct method [15], and as a reference control gene *ACTB* (β-actin) were used.

2.5. Statistics

ImageJ (NIH) program [16] was used for the analysis of Western blot images. We analyzed the Western blot data by setting target protein band and β-actin (control) band values for each sample. Then, density was calculated by dividing target/control and higher density level accepted as one, then the adjusted density values between samples were evaluated. Statistical analyzes were performed for experimental values by One-way ANOVA, two-way ANOVA following Tukey's multiple comparison test and *t-test* (GraphPad Software, Inc., CA, USA) when necessary for the analysis of quantitation of the qRT-PCR, and Western blot. All data are presented as the mean ± SD, with a significance level of $p \leq 0.05$ ($^{*}p < 0.05$, $^{**}p < 0.001$, $^{****}p < 0.0001$).

3. Results

Twenty-two parathyroid hyperplasia tissues were evaluated. The clinical characteristics of the tissue donors were presented in Table 1. Each tissue was isolated enzymatically by the method described previously [17]. The viability profile of parathyroid cells after isolation was determined and the mean cell viability was found 93.3% (mean range 81.8%- 99.8%). In addition, to evaluate the functionality of the isolated cells, $1x10^6$ parathyroid suspension cells were cultivated under stable calcium concentration. The PTH release was found stable for all 22 parathyroid hyperplasia tissues. The levels were between 17.2 and 21.8 pg/mL.

Table 1
Clinical characteristics of parathyroid hyperplasia tissue donor's (n = 22). F: female, M: male. ⚕age at chronic renal failure, [a]type I diabetes mellitus, [b]epilepsy, [c]supernumary parathyroid gland, [d]major depression, n/a: not available.

No	Gender/ age	Age at diagnosis⚕	Dialysis duration (yrs)	Dialysis frequency (per week)	Recurrence (Y/N)	Calcimimetic usage (Y/N)	Serum PTH before surgery (pg/mL)	Other disease (Y/N)
#1	F/42	37	6	2	Y	Y	2175	N
#2	M/33	20	13	3	N	Y	1870	Y[a]
#3	F/32	29	3	3	N	Y	1507	N
#4	M/37	34	3	3	N	N	2553.3	N
#5	F/40	29	11	3	Y	Y	2326	N
#6	M/23	16	7	3	N	N	1378	N
#7	M/40	32	8	3	N	Y	3667.6	N
#8	M/39	29	10	3	Y	N	2154	N
#9	M/38	26	12	3	N	N	860	N
#10	M/42	32	10	3	Y	N	1168.8	Y[b]
#11	F/41	37	4	2	N	N	976	N
#12	M/54	43	11	3	N	Y	2400	N
#13	M/47	37	10	3	N	N	3927	N[c]
#14	M/64	54	10	3	N	Y	4200	Y[d]
#15	F/51	41	10	3	N	N	3120	Y[d]
#16	M/53	43	10	3	N	Y	2150	N
#17	M/52	47	5	3	N	Y	1333	N
#18	F/48	39	9	3	N	N	1690	N
#19	M/60	40	20	3	Y	Y	1150	Y[d]
#20	F/55	47	8	3	N	n/a	1840	N
#21	M/45	41	4	2	N	N	1507	N
#22	F/55	43	12	3	Y	Y	981	N

HLA class II mRNA and protein expression levels (-DR, -DP, -DQ respectively) of 22 parathyroid hyperplasia tissues were determined by qRT-PCR and Western blot. One of the remarkable result is interindividual variation was found different among parathyroid tissues. Firstly, mRNA expression levels showed different patterns between variables ($p = 0.0021$, by one->way ANOVA). When we evaluated by two-way ANOVA following Tukey's multiple comparison tests, we found that only HLA-DP has a high mRNA expression level than -DQ ($p = 0.0017$) (Fig. 1A). However, the protein expression levels altered to a different pattern when compared with the mRNA expressions ($p = 0.0007$ by one-way ANOVA) (Fig. 1B). Based on our data, HLA-DR and -DP showed similar protein expression levels ($p = 0.7874$). The HLA-DQα1 has a higher protein expression level than HLA-DR ($p = 0.0434$) and HLA-DQα2 ($p < 0.0001$). In addition, the HLA-DP protein expression level was found significantly higher than the HLA-DQα2 ($p = 0.0134$). Immunoblot

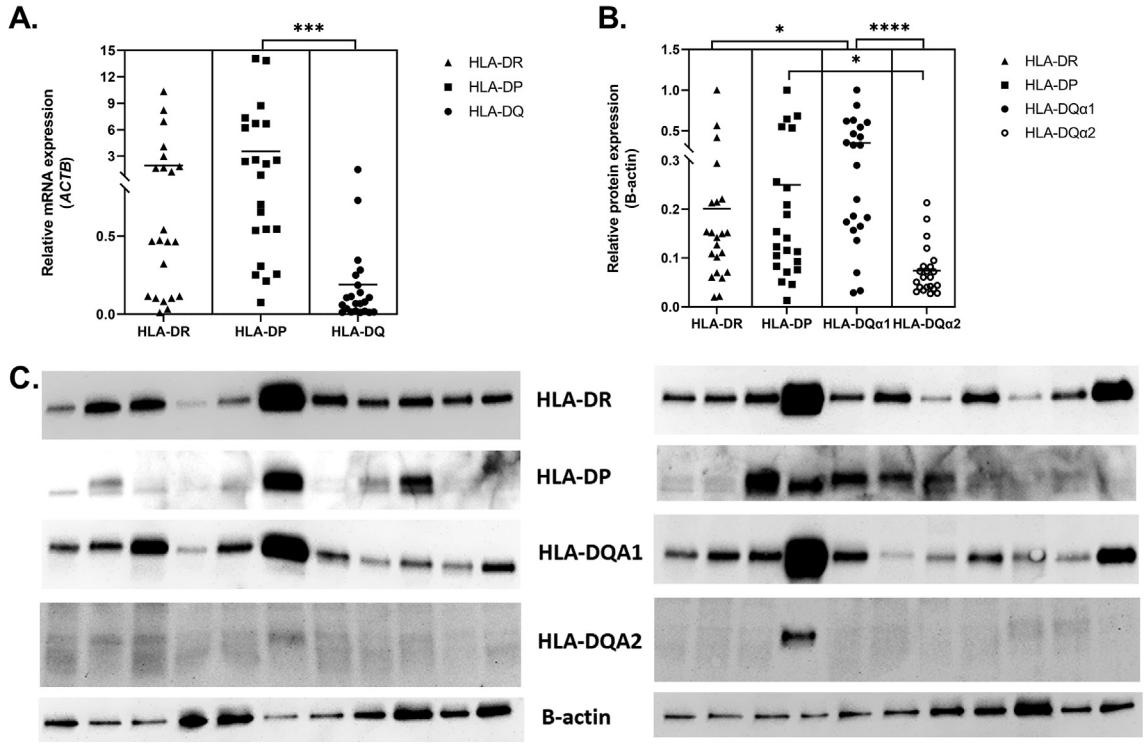

Fig. 1. A. Relative mRNA expression levels (relative to the *ACTB*) by qRT-PCR analysis of HLA-DR, -DP, -DQ respectively in the parathyroid hyperplasia tissues (n = 22). **B.** Relative protein expression levels (relative to the β-actin) by western blot analysis of HLA-DR, -DP, -DQα1, and –DQα2 respectively in the parathyroid hyperplasia tissues (n = 22). **C.** Immunoblot images of HLA-DR, -DP, -DQα1, –DQα2, and β-actin respectively. Samples that were run in different blots were divided and presented in two separate groups for each variable. All data are presented as the mean ± SD, with a significance level of $p \leq 0.05$ (*$p < 0.05$, **$p < 0.001$, ****$p < 0.0001$).

images were compiled in Fig. 1C and the samples that were run in different blots were divided and presented in two separate groups. Statistical analyses were presented as a supplementary file from the output of GraphPad Prism software.

Furthermore, we compared both mRNA and protein expression levels based on the tissue donor characteristics. Regarding the clinical characteristics of the parathyroid donors; four tissue were retrieved from the recurrent operation due to the recurrence of hyperparathyroidism. In addition, five of the patients were reported using calcimimetics [18] to ease the symptoms of hyperparathyroidism. Throughout the evaluation, there were no significant expression changes for HLA class II for both mRNA and protein expression levels while using calcimimetics, or dialysis duration, dialysis frequency or, the recurrence of the hyperparathyroidism. On the other hand, we have divided the parathyroid tissue donors according to their ages including 23 to 42 (indicated as age < 42) and 45 to 64 (indicated as age > 45). As a result, only the HLA-DP mRNA expression level showed higher expression when we compared the age groups (Fig. 2).

4. Discussion

Parathyroid allotransplantation (PA) is the only curative treatment option for patients who have permanent hypoparathyroidism [19]. Since 1990, significant efforts have been made for parathyroid cell allotransplantation mostly by two groups in the literature. First, Nawrot et al. reported 85 transplantation cases in 2007 [7], and subsequently, the same group reported the highest cohort (n = 316) of parathyroid transplantation between 1996 and 2017 with a varying survival rate [6]. Second, Aysan et al. reported the second-highest clinical cases (n = 44) [20]. Thereafter, Yucesan et al. carried on with the determination of pre-op and post-op immunological criteria for parathyroid allotransplantation [19,21].

One of the outcomes of allotransplantation is the levels of alloantibodies against the donor HLA complex (protein products) [22]. The term tissue immunogenicity refers to the main mechanism which is directly regulated by the antigen-presenting cells, to present alloantigens from the transplanted graft. Therefore, the tissue-specific immunogenicity is related to the HLA molecules

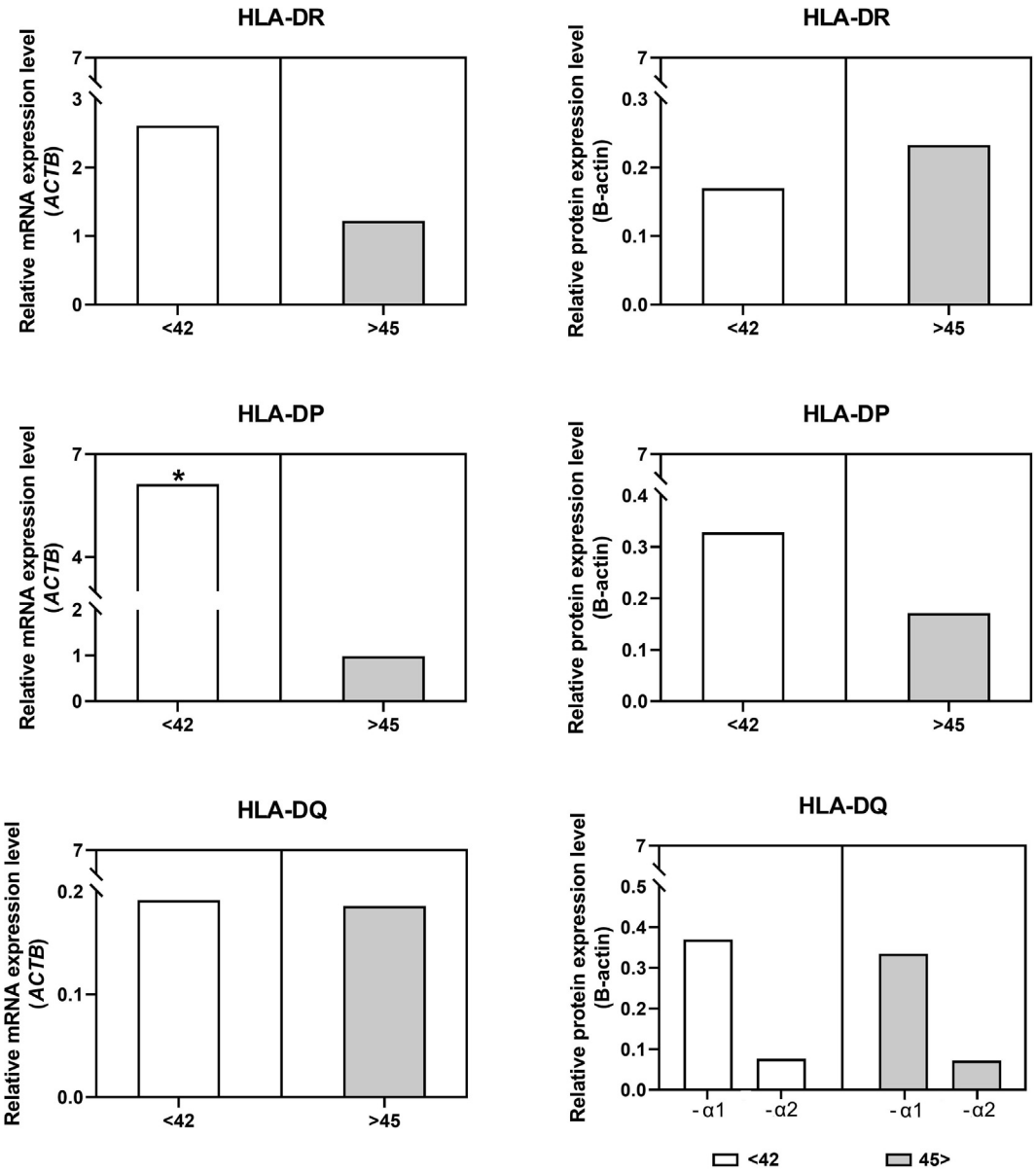

Fig. 2. Representation of the relative mRNA (left side) and protein (right side) expression changes of HLA-DR, HLA-DP, and HLA-DQ (-α1 and -α2) respectively. A comparison of relative expression levels was presented between age under 42 and age over 45 for each variable. * indicates $p < 0.05$.

of the graft [14,23]. Another issue regarding immunosuppression regimens is regularly used during major organ transplantations and the follow-up processes and continue mostly for life-long [22,24]. Although, the effect and side effects of immunosuppression are not acceptable for parathyroid transplant recipients. Besides, calcium and active vitamin D replacement therapy are more reasonable to take. As is known, the PA is the first and only curative option for permanent hypoparathyroidism. Permanent hypoparathyroidism is mostly reported as a rare disease [25] however a significant increase in papillary thyroid cancer occurred after Chernobyl disaster around closest regions. The treatment requires thyroidectomy which may indirectly causes permanent hypoparathyroidism. Therefore understanding the parathyroid immunogenicity is important and transplantation is a priority for most of the patient population for those in need.

Manipulation of cell surfaces may impact the recipient's immune system interaction with HLAs. Innovative methods were improved and presented clinically by Tolloczko et al., Nawrot et al., respectively. They both used IFN treatment during four to six weeks of cultivation, cryopreservation, then particular parathyroid cell selection which only included low expressed HLA class I and II molecules before transplantation [7,8]. Another technique was reported by Tsuji et al. in 1999 and they presented four weeks of cultivation process which included cryopreservation + cultivation to ease parathyroid hyperplasia tissue antigenicity [11]. Besides, such treatments were also reported for endothelial cells e.g. treatment with anti-blood group antibodies to lower HLA-DR expression to prevent complement system-mediated cytotoxicity [26]. Although, it is not possible to predict if one antibody will show a positively correlated effect on the various cell surface antigens.

Researchers and clinicians mostly aimed to prepare parathyroid cells for allotransplantation and lower HLA expressions before transplantation. Despite the number of transplantations and published cell preparation/cultivation methods, there is still a need for improvements for longer graft survival. Besides, parathyroid transplantation requires finding a suitable donor who has chronic renal failure and who is referred for subtotal parathyroidectomy as well. However, based on our experience from the literature and our works, there are certain limitations such as finding a pathologically suitable donor, determination of cell isolation process to keep tissue viable and, functional as long as possible, and the feasible cell amount for transplantation. Longer cultivation of parathyroid cells leads to lower viability and then only a limited number of live cells remain [6,11]. While there is a comprehensive selection of parathyroid cell preparation reported so far, there is no common consensus between methods. Therefore in our transplantation unit, we did not prefer longer cultivation due to the possibility of a loss of function, instead, the pre-op immunological criteria between donor and recipient showed more improved results [21,27].

Another pertinent consideration about HLA class I, and II molecules in parathyroid hyperplasia tissue is mostly reported as non-immunogenic with low HLA class I expression therefore it is not involved in the rejection process after transplantation [6]. However, in 2019 Goncu et al. showed varying degrees of expression in HLA class I molecules (-A, -B, and -C for both mRNA and protein expression) and stable HLA-A expression in parathyroid hyperplasia tissue [14]. Later in this study, in 2021 Goncu et al. have performed an allotransplantation between HLA-A allele matching recipient and a donor. Then followed the clinical outcome for almost a year and the graft survival continues [27]. Based on our findings for HLA class I molecules both experimentally and clinically, cause us to question the state of the HLA class II expression for parathyroid tissue for donor selection criteria. In addition, after four years of transplantation follow-up, one of the five recipients showed positive donor-specific antibodies (DSA), against HLA class I [21], and this finding is considered as a possible outcome of the stable protein expression for HLA-A of the parathyroid tissue. As is known, the HLA-DQ regarding the developing *de novo* DSA after transplantation has been reported for heart and kidney transplantations [28-30]. As is shown by others, after transplantation the most common antibodies are anti-HLA-DQ, thus the presence of HLA-DQ antigens should be taken into account.

Therefore, to investigate HLA class II expression of tissue donors, we evaluate HLA-DR, -DP, -DQ mRNA and HLA-DR, -DP, -DQ α1 and α2 total protein expression levels in parathyroid cells. As a result, the donor's clinical characteristics including dialysis duration, dialysis frequency, the recurrent donation from the same donor due to hyperparathyroidism and, calcimimetic usage before the donation were not found significant between parathyroid tissue samples. Despite there is no obvious significance with the clinical characteristics when we compared the HLA class II expression, the age of the donor could be a crucial factor with or without HLA class II expression levels. Aging is a physiological process, and recent studies reported various biological markers about age [31], thus the age of the parathyroid donor may affect several functions that are not yet known. On the other hand, we found that HLA-DP has high mRNA expression levels in the age below 42 groups and this higher expression did not affect the protein level. Interestingly, HLA-DR, HLA-DP, and HLA-DQ α1 protein expression showed permanent and varied expression rates between tissues. Particularly, the HLA-DQ mRNA expression levels were found less expressed while the protein levels of -DQα1 showed higher expression when compared to -DQα2. We acknowledge that there are some limitations in this study which has a limited sample size, the lack of particular ages 43 and 44 among parathyroid donors, and there was no clinical outcome to test our conclusion yet. In addition, first in the literature, this study provides the HLA-DQ-α chain expression profile of parathyroid tissue. The HLA-DQ-β chain expression status was reported as not detected in the Human Protein Atlas database [32]. Alternatively, the status of the other HLA class II molecules should be an encounter for future studies.

There is neither consensus nor elucidation between allele matching criteria for PA yet. The current study further explores the understanding of the parathyroid tissue-specific immunogenicity and particularly, provides a basis for the allotransplantation allele criteria assessment. However, when performing PA, the rejection of the graft could be due to these HLA expression levels. Nonetheless, there are still several questions that need answers. Among them; would such HLA expression levels require an exact match for HLA allele matching? Is there a possible limit for PA such as HLA-A, -B, -DR allele matching like in kidney transplantation? If not, what will be the frequency of *de novo* antibody follow-up and/or formation after this adaptation?

Since 2013 our team has been performing PA and to the best of our knowledge, we presented the immunological criteria (pre-op assessment and post-op follow-up) [19,21,33] to maintain/improve clinical outcomes for permanent hypoparathyroidism patients first in literature. In the last eight years, we continue using research data to expand our clinical improvements. The best donor for the best recipient limit remains to be elucidated.

In conclusion, this study demonstrates detailed levels of HLA class II molecules which could represent a potential criterion before determining a transplantable parathyroid graft. Further studies about PA with a longer observation period and suitable parathyroid donor criteria to increase the survival rate of transplantation are required.

5. Ethics statement

This study was conducted after receiving approval from the Local Human Ethics Committee (approval number: 71306642–

050.01.04). All of the protocols conformed to the ethical guidelines of the Helsinki Declaration and written informed consent was obtained from all subjects.

6. Data availability statement

The data described in this article are openly available within the article and in the supplementary files. Raw data that support the findings other than the included of this study are available from the corresponding author upon reasonable request.

CRediT authorship contribution statement

Beyza Goncu: Conceptualization, Methodology, Data curation, Software, Writing – original draft, review & editing. **Emrah Yucesan:** Data curation, Writing – original draft. **Yeliz Emine Ersoy:** Supervision, Visualization, review & editing. **Mustafa Erhan Aysan:** Supervision, Visualization. **Nur Ozten Kandas:** Supervision, Visualization, review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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