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Short report

High-resolution melting analysis to screen the *ST18* gene functional risk variant for pemphigus vulgaris: The occasion to open a debate on its usefulness in clinical setting

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ABSTRACT

The *ST18 -497-65050 T > C* polymorphisms (rs17315309) exhibit a very strong association in the pathogenesis of Pemphigus Vulgaris (PV) and could represent a new potential molecular target for the treatment of disease.

The present study aimed to establish a low-cost, sensitive and reliable assay using high-resolution melting curve analysis (HRMA) on magnetic induction rotor-based platform, the Magnetic Induction Cycler (MIC) (Bio molecular Systems).

HRMA assay was able to identify easily and unambiguously the *c.-497-65050 T > C* genotypes evaluating melting curve shape and melting temperature (T_m). The results of HRMA were validated by direct DNA sequencing.

The HRMA is rapid, sensitive, low-cost and high-throughput assay to screen the *rs17315309* variant and could be used in clinical diagnostic laboratories.

1. Introduction

Pemphigus describes a group of autoimmune, mucocutaneous blistering disorders characterized by production of IgG autoantibodies against extracellular domains of cell membrane proteins of keratinocytes resulting in acantholysis (Bystryn and Rudolph, 2005; Sinha, 2011; Kasperkiewicz et al., 2017).This rare skin disease can be classified into three major forms: pemphigus vulgaris, pemphigus foliaceus and paraneoplastic pemphigus.

The estimated incidence of pemphigus vary substantially around the world. Among the three forms, pemphigus vulgaris (PV) is the most frequent and representative form of the group with an annual incidence of 0.76–6.7 new cases per million worldwide (Bystryn and Rudolph, 2005) and it is between 4-to10-fold more common among Jews as compared with other populations (Ruocco et al., 2013).

In PV, the principal type of autoantibodies produced are against Desmogleina 3, resulting in loss of cell-cell adhesion within the epidermis (Ruocco et al., 2013; Stanley and Amagai, 2006). More recently, a number of additional pathogenic mechanism have been suggested to be operative in PV, such as increased cell apoptosis, aberrant cell-cell signaling leading to collapse of the cytoskeleton of basal keratinocytes with consequent shrinkage of these cells, actin reorganization and activation of muscarinic receptors uniquely expressed on basal cells (Grando, 2006; Grando et al., 2009; Chernyavsky et al., 2007).

The existence of a complex polygenic basis, involving multiple genetic loci, is clearly established for PV (Sinha, 2011). A number of candidate gene-driven case-controls studies conducted in several populations have revealed an association between PV and human leukocyte antigen (HLA) class I and II regions. Currently, a non-classical class IB allele and a non-HLA genes have been definitively linked to PV

Abbreviations: PV, Pemphigus Vulgaris; HLA, Human Leukocyte Antigen; HRMA, High Resolution Melting Analysis; T_m, Melting Temperature; CV, Coefficient of Variation

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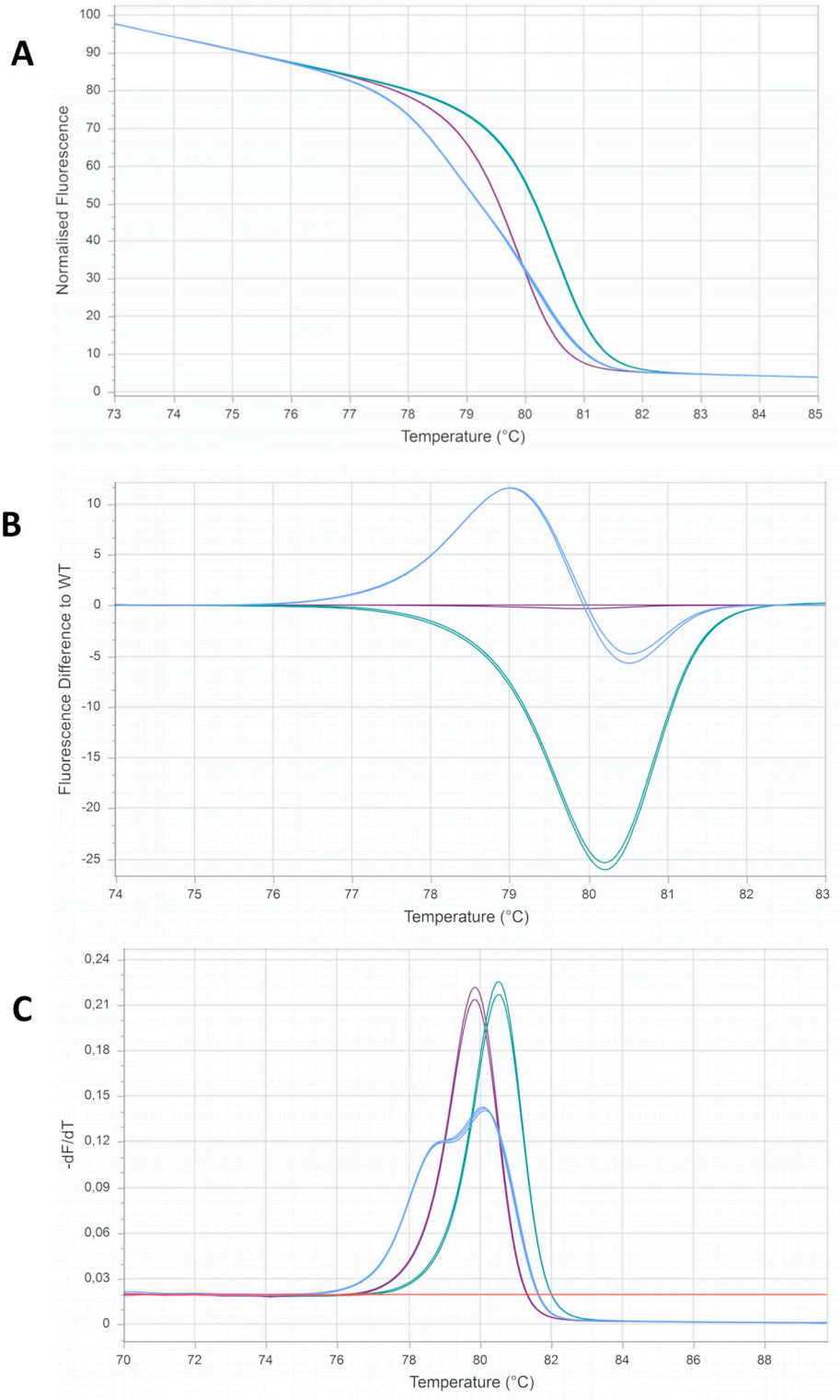

Fig. 1. Normalized and Temperature-shifted Plot (A), Fluorescence Difference Plot (B) and Melting Peaks (C) of the three genotypes profiles of *ST18* c.*-497-65050 T > C*. Data were reported for wild type (violet), homozygous (green) and heterozygous (blue). For the homozygous sample we observed a melting temperature shift of approximately of 0.7 °C, while heterozygous profiles show different melting profiles with double peaks in the Derivative Plot. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
The evaluation of Melting Temperature (T_m) for each genotypes of the *rs17315309* SNP.

Rs17315309 genotype	T_m (°C) ± SD
Wild-type	79.97 ± 0.05
Heterozygous	78.78 ± 0.04/80.18 ± 0.1
Homozygous	80.59 ± 0.01

(Sinha, 2011; Kasperkiewicz et al., 2017; Tron et al., 2005). In particular, Dan Vodo et al. (2016) have identified a specific variant within the promoter of *ST18* gene (namely *rs17315309*) that was associated to higher risk of disease development. The presence of risk allele C (regardless of heterozygote or homozygote status) may drives gene transcription in a p53/p63-dependent manner, resulting in an up-regulation of inflammatory molecules as TNFα, IL-1α and IL-6 which, in turn, can contribute to apoptosis and inflammation, specifically influencing the susceptibility of epidermal cells to PV-IgG-induced acantholysis. Therefore, these results support a direct role for *ST18* in the pathogenesis of PV and, consequently, highlighting this variant as a new potential molecular target for the treatment of disease (Vodo et al., 2016; Sarig et al., 2012).

In order to provide a rapid and reliable tool for the screening of *rs17315309* variant in patients with PV, we applied the High-Resolution Melting Analysis (HRMA) (Montgomery et al., 2010; Er and Chang, 2012; Słomka et al., 2017). This method is proven to be simple, rapid and low-cost for SNP scanning, making it ideal in both molecular research and diagnostics settings (De Paolis et al., 2018; Minucci et al., 2017a; Minucci et al., 2017b).

The aim of this study is to set up a HRMA method using the Magnetic Induction Cycler (MIC) (Bio molecular Systems), an innovative magnetic induction rotor-based platform recently become commercially available, to screen the *ST18* rs17315309 variant in patients with PV addressed to our laboratory.

2. Materials and method

2.1. Subjects and DNA extraction

Three *ST18* genotypes (wild-type, heterozygous and homozygous,) previously characterized by direct sequencing, were used as standard for preliminary HRMA set up.

In addition, HRMA was evaluated on 25 further DNAs, including 5 patients with diagnosis of PV and 20 DNA healthy volunteers, who were randomly selected within our biobank (Etichal Committee authorization: Register CE: 384; 56/CE/2017). The patients satisfied the typical clinical, histological and immunopathological features of PV; in particular they showed an intercellular staining pattern of IgG and C3 by direct immunofluorescence and the presence of Abs anti Dsg1 and/or Dsg3.

Genomic DNA was extracted from peripheral blood samples using an automated method based on commercial kit (QIAamp® mini DNA extraction kit, QIAGEN) according to manufacturer's procedures.

DNA quantity and quality were checked by Qubit dsDNA BR Assay kit (ThermoFisher Scientific), while DNA integrity was verified by 0.8% agarose gel electrophoresis.

2.2. Assay design

Primers were designed by Primer3 software v4.1.0 (http://primer3.ut.ee) to amplify a small fragment surrounding the c.*-497-65050 T > C (rs17315309)* variant, based on NC_000008.11 as reference sequence. These primers were purified by HPLC and certified as high molecular quality products (Bio-Fab research, Rome, Italy, http://www.biofabresearch.it). The 72-bp PCR products were amplified using the following primers: 5′-ATCACCTCTAGCCTGACAAAC-3′ (forward) and 5′-AAGTGGTTACACGTGTGACG-3′ (reverse). The folding characteristic of PCR products and primers were determined using the secondary structure profiling software DINAMelt (http://unafold.rna.albany.edu/?q=DINAMelt). In order to test the specificity of primers' sequences (https://blast.ncbi.nlm.nih.gov/Blast.cgi), BLAST software was used. With the purpose to select the optimal annealing temperature (T_a) for our assay, we performed a gradient PCR within the range of ± 10 °C using the median temperature from the T_a range proposed by Primer3 as the starting point. PCR products were then analyzed by 4% agarose gel electrophoresis to detect spurious bands. Only the T_a providing a single PCR product (72 bp in length) was selected.

2.3. HRM conditions

PCR and HRMA were performed in a single tube on Mic platform (Bio Molecular Systems).

Reaction volume was 20 µL: 3 µL of genomic DNA (10 ng/µL) was added to 10 µL of LightCycler® 480 High-Resolution Melting Master 2× (including buffer, Taq polymerase, nucleotides and the proprietary dsDNA saturating binding dye), with 2.5 mM of $MgCl_2$ and 0.15 µM of forward and reverse primers. These mix were then placed into reaction tubes prefilled with oil (Bio Molecular Systems).

The PCR program started with an initial denaturation of 10′ at 95 °C, continued with 40 cycles of 10″ at 95 °C, 15″ at 56 °C and 2′ at 72 °C. For HRMA, PCR products were then heated by the three following steps: denaturation at 95 °C for 1′, renaturation at 40 °C for 1′ and second denaturation from 65 °C to 95 °C with a ramp rate of 0.1 °C/s collecting 25 acquisition/°C.

All samples were tested in duplicate and a negative (DNA-free blank) reaction was run in order to check possible contamination within reagents.

2.4. HRM analysis

At the end of the run (approximately ninety minutes), the analysis of melting curves was performed using the MIC qPCR Software. Samples genotyping was obtained in two steps. The first step is based on the normalization of the raw melting curve data by setting the pre-melt (initial fluorescence) and post-melt (final fluorescence) signals of all samples to unique uniform values. Pre-melt signals are set to a relative value of 100%, while post-melt signals are set to a relative value of 0%. The optimal normalization setting was obtained with a pre-melting of 74–75 °C and a post-melting of 82–83 °C.

The three reference genotypes (wild-type, heterozygous and homozygous) were set in the second analysis step, allowing the evaluation of the differences in melting curve shapes through the “Difference Plot”. The software will automatically classify each unknown samples against a reference genotype, comparing and matching the unknown curve to the nearest reference curve.

Finally, the Melt Analysis tool calculates the T_m values using the melting peaks obtained as derivative curve plotted as -dF/dT *vs* temperature.

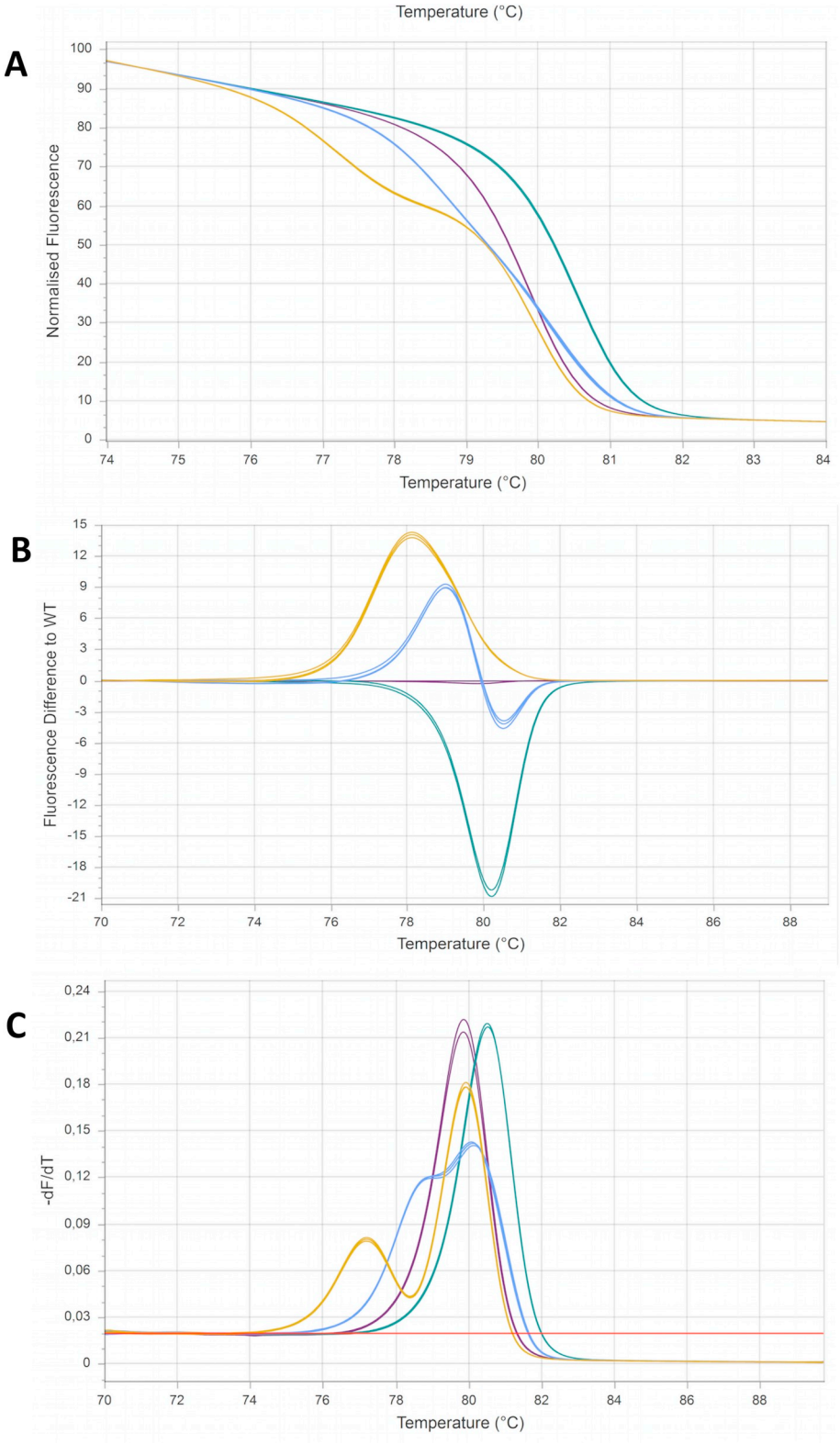

Fig. 2. Normalized and Temperature-shifted Plot (A), Fluorescence Difference Plot (B) and Melting Peaks (C) of one PV sample with different melting curve behavior (yellow profile) are shown. This sample reveal a different and clearly distinguishable melting profile than the three standard genotypes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
The *ST18* promoter genotype of PV cases.

Case number	*Rs17315309* genotype
PV_1	*TT*
PV_2	*TC*
PV_3	*TT*
PV_4	*TT*
PV_5	*TC* (with *c.-496-65050G > A*)[a]

[a] Compound heterozygosis.

2.5. Validation of HRM method

To validate the pipeline, each samples analyzed by HRM was genotyped by Sanger sequencing as confirmatory test. A separate PCR was carried out to generate a larger product (180-bp) using the following primers: 5′-GCTTGCCGTTTGTAAGATG-3′ (forward) 5′-AAGTGGTTAC ACGTGTGACG-3′ (reverse) (Bio-Fab research, Rome, Italy, http://www.biofabresearch.it). PCR products were cleaned with ExoSAP-IT® (Affymetrix Inc., Santa Clara, USA) and subsequently bidirectionally sequenced using the BigDye Terminator v3.1 Cycle Sequencing kit on the Applied Biosystems 3130 Genetic Analyzer (Thermo Fisher Scientific). The data were then analyzed with both SeqScape® software v.3 and Sequencing Analysis Software v.6 (Thermo Fisher Scientific).

3. Results

3.1. HRMA analysis

The HRMA screening was performed *"in blind"* on 25 samples, 5 PV-cases and 20 DNA healthy volunteers. We were able to clearly genotype all samples by evaluating their specific melting profiles (Fig. 1) along with the T_m values (Table 1).

The melting peak for homozygous TT samples occurs at a lower temperature than the homozygous CC one. In TC heterozygotes, both temperature peaks can be detected. Homozygote genotypes for the variant were easily identified by a T_m shift of 0.7 ± 0.03 °C compared to the wild-type ones.

Among the 5 PV DNA samples, we obtained the following genotypes: 3 wild-type, 1 heterozygous for the *c.-497-65050 T > C* variant and 1 unclassified. Particularly, the last one presented a melting curve behavior and a T_m value clearly different to the three standard *ST18* genotypes (Fig. 2). The latter was subsequently tested by direct Sanger sequencing in order to identify the specific genotype, as followed discussed (Table 2).

However, among the 20 DNA healthy volunteers, we found 15 wild-type and 5 heterozygotes. Although not significantly different between our cases and controls, our results are not superimposable to those previously reported (Vodo et al., 2016).

3.2. Validation of HRMA

To validate the assay, all HRMA results were verified by direct DNA sequencing, that confirmed all the detected genotypes. Regarding the sample showing a difference in melting profile from references, Sanger sequencing identified a compound heterozygosis consisting of *rs17315309* variant combined with another missense nucleotide substitution (*c.-496-65050G > A*) within the *ST18* promoter region (Fig. 3).

Our results indicate 100% concordance between sequencing analysis and HRMA genotyping.

3.3. Sensitivity of HRMA

We evaluated the intra-assay precision analyzing four replicates of one single wild-type and homozygous/heterozygous samples, respectively. Furthermore, the inter-assay precision was assessed running the same wild-type, homozygous and heterozygous samples in triplicates on 3 different PCR/HRMA experiments. We achieved a full superimposability and reproducibility of melting profiles and T_m. In terms of repeatability we obtained T_m CV ≤0.4% with a SD < 0.1%, while the reproducibility for T_m CV resulted as ≤0.5% with a SD < 0.1%.

In order to test the sensitivity of HRMA, we evaluated serial dilutions (1:2, 1:4, 1:8, 1:16, 1:32) of wild-type, homozygous and heterozygous DNAs, starting from 15 ng/μL. Melting curves of positive samples were still well differentiated as compared to wild-type ones, until the lowest variant allele frequency investigated (1:32; data not shown).

4. Discussion

HRMA was introduced in molecular diagnostic laboratories as valid technique characterized by several advantages: cost-effectiveness, efficiency and absence of pre/post-PCR treatment (Montgomery et al., 2010). Furthermore, it is a simple approach for the genotyping and/or mutation scanning (Er and Chang, 2012). These characteristics, together with the possibility to analyze up to 96 sample in a short time, make HRMA suitable for high throughput applications.

Herein, we describe the development of a sensitive and reliable HRMA assay for the scanning of *rs17315309* variant within the region promoter of *ST18* gene in patients affected with PV.

PV is the most frequent and representative form of autoimmune bullous disease with intraepithelial lesions involving the skin and the Malpighian mucous membranes (Ruocco et al., 2013).

Many evidences support the role of genetics factors in PV development. Recently, by a genome-wide association study, the risk variant *rs17315309* within the *ST18* promoter region was identified. This PV-associated risk allele may drive *ST18* up-regulation, which in turn could contribute to PV pathogenesis by stimulating keratinocyte-derived cytokine release and by compromising epidermal cell-cell adhesion (Vodo et al., 2016; Sarig et al., 2012). Definitively, previous studies underscored the importance of genetic variations affecting target tissues in the pathogenesis of inflammatory diseases: among these, the *rs17315309* seems to play an important role in PV pathogenesis.

Nevertheless, the complexity of the molecular mechanisms surrounding this disease still represents a challenge, above all in terms of patients' screening and diagnosis.

Besides the classic routine assays (autoantibodies against Desmoglein 3 and such HLA class II alleles) the presence of other susceptibility factors should be taken into account. Therefore, following the literature suggestions (Vodo et al., 2016), to better stratify risk and improve the diagnostic workflow, many dermatologists are interested in testing PV patients attending the reference Dermatology Units for this potential biomarker, as more information can contribute altogether to a better patients' evaluation.

Therefore, the number of patients needing this assay is being increasing in our laboratory. For this reason, we decided to set up a reliable, robust and cheaper assay able to satisfy clinical and laboratory requirements.

Furthermore, this HRM approach represents an example of a simple and less expensive tool, useful in the clinical laboratory routine *ST18* genotyping.

Surprisingly, we found as the frequency of C allele in *ST18* gene was higher in controls than in PV patients. This finding could be due to the different ethnic origin of our patient as compared to the study of Vodo et al. (2016), where only Jewish PV individuals were enrolled. Contrastingly our PV patients were all Caucasian.

Therefore, by means of our HRMA approach, it would be really easier to extend this type of assay on larger cohorts of both controls and patients, in order to verify the usefulness of this biomarker in clinical setting.

Although PV is a rare disease, the main limitation of our study is

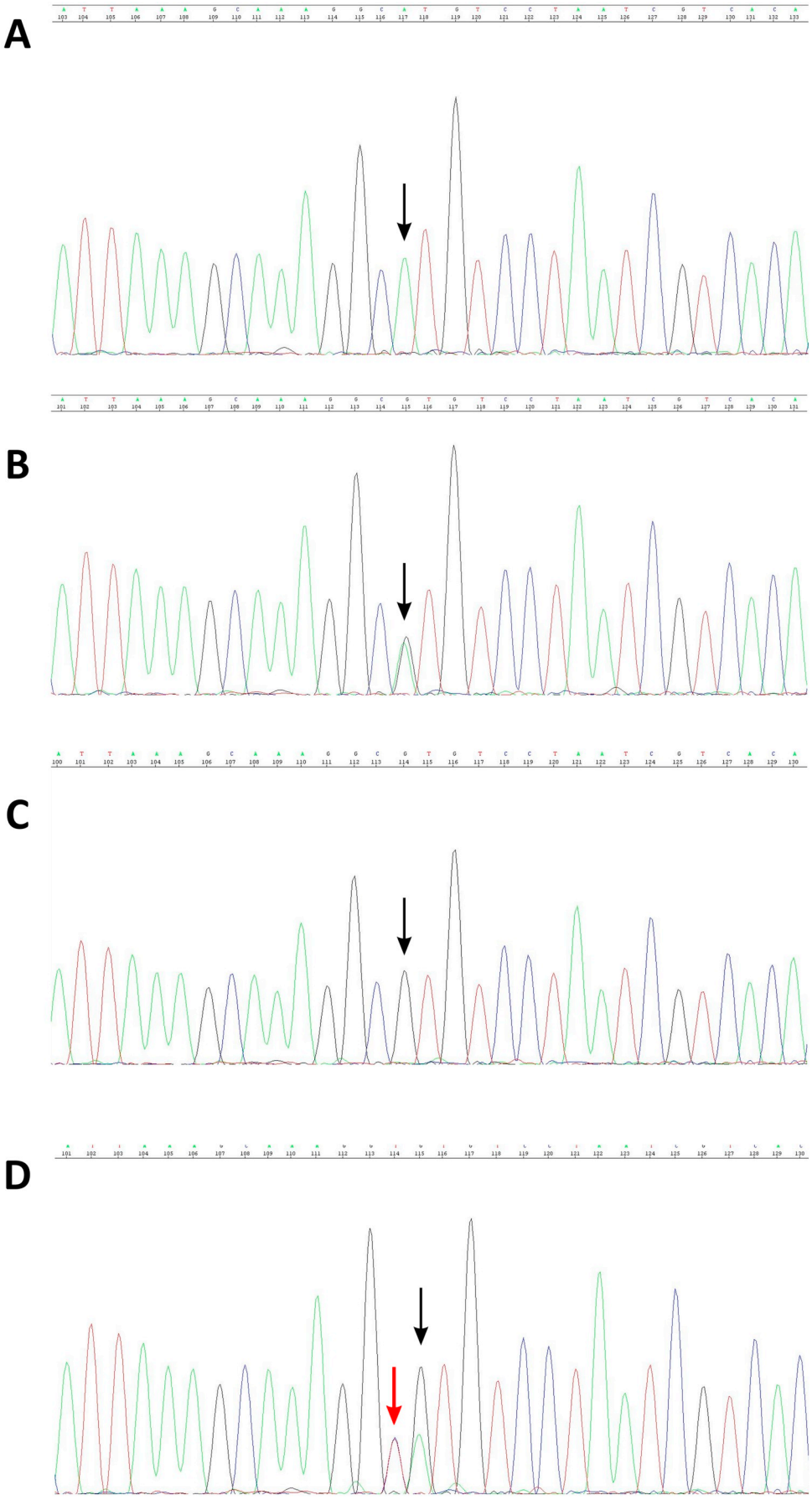

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Fig. 3. Sanger sequencing results for the target variant *c.-497-65050 T > C (rs17315309)*. Partial chromatograms of the four genotypes (A, wild type; B, heterozygous; C, homozygous; D, compound heterozygous) collected in this study are herein reported. The black arrows note the location of the variant of interest, while the red arrow indicates the new variant found in unknown sample. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

represented by the relative small sample size: we underline as the aim of the present report is the set-up of HRMA method rather than an epidemiological study on prevalence of *rs17315309* variant in a PV patient cohort.

Nevertheless, further studies, involving larger sample sizes, should be carried out before it can be routinely implemented in clinical setting.

However, using of our HRMA approach, which is cheaper, easy and rapid to use, could facilitate large cohort studies on *ST18 rs17315309* variant in order to determine the impact of this variant in the disease development.

Conflict of interest

All authors (MDB, EDP, GLS, LF, MML, CM, GDZ, AM and EC) have read and approved submission of the manuscript. The paper has not been published and is not being considered for publication elsewhere in whole or part in any language. All authors declare that there is no conflict of interests regarding the publication of this paper.

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References

Bystryn, J.C., Rudolph, J.L., 2005. Pemphigus. Lancet 366 (9479), 61–73. https://doi.org/10.1016/S0140-6736(05)66829-8.

Chernyavsky, A.I., Arredondo, J., Piser, T., Karlsson, E., Grando, S.A., 2007. Differential coupling of M1 muscarinic and alpha7 nicotinic receptors to inhibition of pemphigus acantholysis. J. Biol. Chem. 283 (6), 3401–3408. https://doi.org/10.1074/jbc.M704956200.

De Paolis, E., Minucci, A., De Bonis, M., Scaglione, G.L., Gervasoni, J., Primiano, A., Ferraro, P.M., Cappellani, D., Marcocci, C., Gambaro, G., Capoluongo, E., 2018. A rapid screening of a recurrent CYP24A1 pathogenic variant opens the way to molecular testing for Idiopathic Infantile Hypercalcemia (IIH). Clin. Chim. Acta 482, 8–13. https://doi.org/10.1016/j.cca.2018.03.024.

Er, T.K., Chang, J.G., 2012. High-resolution melting: applications in genetic disorders. Clin. Chim. Acta 414, 197–201. https://doi.org/10.1016/j.cca.2012.09.012.

Grando, S.A., 2006. Cholinergic control of epidermal cohesion. Exp. Dermatol. 15 (4), 265–282. https://doi.org/10.1111/j.0906-6705.2006.00410.x.

Grando, S.A., Bystryn, J.C., Chernyavsky, A.I., Frusić-Zlotkin, M., Gniadecki, R., Lotti, R., Milner, Y., Pittelkow, M.R., Pincelli, C., 2009. Apoptolysis: a novel mechanism of skin blistering in pemphigus vulgaris linking the apoptotic pathways to basal cell shrinkage and suprabasal acantholysis. Exp. Dermatol. 18 (9), 764–770. https://doi.org/10.1111/j.1600-0625.2009.00934.x.

Kasperkiewicz, M., Ellebrecht, C.T., Takahashi, H., Yamagami, J., Zillikens, D., Payne, A.S., Amagai, M., 2017. Pemphigus. Nat. Rev. Dis. Primers 11 (3), 17026. https://doi.org/10.1038/nrdp.2017.26.

Minucci, A., De Bonis, M., De Paolis, E., Gentile, L., Santonocito, C., Concolino, P., Mignone, F., Capoluongo, E., 2017a. High resolution melting analysis is very useful to identify BRCA1 c.4964_4982del19 (rs80359876) founder Calabrian pathogenic variant on peripheral blood and buccal swab DNA. Mol. Diagn. Ther. 21 (2), 217–223. https://doi.org/10.1007/s40291-017-0262-3.

Minucci, A., De Paolis, E., Concolino, P., De Bonis, M., Rizza, R., Canu, G., Scaglione, G.L., Mignone, F., Scambia, G., Zuppi, C., Capoluongo, E., 2017b. Competitive PCR-High Resolution Melting Analysis (C-PCR-HRMA) for large genomic rearrangements (LGRs) detection: a new approach to assess quantitative status of BRCA1 gene in a reference laboratory. Clin. Chim. Acta 470, 83–92. https://doi.org/10.1016/j.cca.2017.04.026.

Montgomery, J.L., Sanford, L.N., Wittwer, C.T., 2010. High-resolution DNA melting analysis in clinical research and diagnostics. Expert. Rev. Mol. Diagn. 10 (2), 219–240. https://doi.org/10.1586/erm.09.84. (Review. PubMed PMID: 20214540).

Ruocco, V., Ruocco, E., Lo Schiavo, A., Brunetti, G., Guerrera, L.P., Wolf, R., 2013. Pemphigus: etiology, pathogenesis, and inducing or triggering factors: facts and controversies. Clin. Dermatol. 31 (4), 374–381.

Sarig, O., Bercovici, S., Zoller, L., Goldberg, I., Indelman, M., Nahum, S., Israeli, S., Sagiv, N., Martinez de Morentin, H., Katz, O., Baum, S., Barzilai, A., Trau, H., Murrell, D.F., Bergman, R., Hertl, M., Rosenberg, S., Nöthen, M.M., Skorecki, K., Schmidt, E., Zillikens, D., Darvasi, A., Geiger, D., Rosset, S., Ibrahim, S.M., Sprecher, E., 2012. Population-specific association between a polymorphic variant in ST18, encoding a pro-apoptotic molecule, and pemphigus vulgaris. J. Invest. Dermatol. 132 (7), 1798–1805. https://doi.org/10.1038/jid.2012.46.

Sinha, A.A., 2011. The genetics of pemphigus. Dermatol. Clin. 29 (3), 381–391. https://doi.org/10.1016/j.det.2011.03.020.

Słomka, M., Sobalska-Kwapis, M., Wachulec, M., Bartosz, G., Strapagiel, D., 2017. High Resolution Melting (HRM) for high-throughput genotyping-limitations and caveats in practical case studies. Int. J. Mol. Sci. 3 (18), 11. https://doi.org/10.3390/ijms18112316.

Stanley, J.R., Amagai, M., 2006. Pemphigus, bullous impetigo, and the staphylococcal scalded-skin syndrome. N. Engl. J. Med. 355 (17), 1800–1810. https://doi.org/10.1056/NEJMra061111.

Tron, F., Gilbert, D., Mouquet, H., Joly, P., Drouot, L., Makni, S., Masmoudi, H., Charron, D., Zitouni, M., Loiseau, P., Ben Ayed, M., 2005. Genetic factors in pemphigus. J. Autoimmun. 24 (4), 319–328. https://doi.org/10.1016/j.jaut.2005.03.006.

Vodo, D., Sarig, O., Geller, S., Ben-Asher, E., Olender, T., Bochner, R., Goldberg, I., Nosgorodsky, J., Alkelai, A., Tatarskyy, P., Peled, A., Baum, S., Barzilai, A., Ibrahim, S.M., Zillikens, D., Lancet, D., Sprecher, E., 2016. Identification of a functional risk variant for pemphigus vulgaris in the ST18 gene. PLoS Genet. 12 (5). https://doi.org/10.1371/journal.pgen.1006008.