EJGO European Journal of Gynaecological Oncology

Novel CARD14 mutations in Chinese samples with ovarian endometriosis

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Summary

Objectives: Endometriosis is a potential pre-malignant gynecological condition harboring multiple gene mutations in the endometriotic lesions. Evidence has implicated the possibility that caspase recruitment domain family member 14 (CARD14) mutations might exist in ovarian endometriosis. It is questioned whether ovarian endometriosis harbors CARD14 mutations. *Materials and Methods:* The authors recruited and analyzed a cohort of 101 ovarian endometriosis samples for the presence of CARD14 mutations via sequenced the coding and intron/exon boundary regions of the CARD14 gene. *Results:* A novel heterozygous somatic CARD14 mutation, p.R470C (c.1408C>T), was identified in two out of 101 (2.0%) endometriotic lesions. The mutated samples were 30- and 31-years-old and both had a history of childbirth. No CARD14 mutations were detected in the remaining 99 samples with ovarian endometriosis. Protein structure modeling results showed CARD14 p.R470C mutation resulted in protein structural changes. These combined results implicated that this mutation might be damaging. However, no association was observed between the main clinical features and CARD14 mutations in this sample cohort. *Conclusion:* The authors identified a potential damaging CARD14 somatic mutation in 2/101 (2.0%) endometriotic lesions with ovarian endometriosis for the first time, and this mutation might play an active role in the development of ovarian endometriosis.

Key words: CARD14; Mutation; Ovarian endometriosis; Chinese.

Introduction

Endometriosis is a common estrogen-dependent chronic gynecological disease characterized by the growth of the endometrial glands and stroma outside the lining of the uterus, exhibiting frequently chronic pelvic pain, dyspareunia, severe dysmenorrhea, and infertility [1-3]. Endometriosis could be subdivided into three different subtypes, peritoneal endometriosis, ovarian endometriosis, and deeply infiltrating endometriosis [4], despite endometriosis is usually regarded as a benign disorder, malignant transformation may occur in approximately 1% of cases, especially commonly in ovarian lesions, usually harboring somatic mutations in multiple genes [5-8]. To date, the detailed pathogenesis of endometriosis remains not fully understood, and multiple theories have been proposed, among which, the widely accepted theories implicated that genetic, biochemical, or immunological dysfunction of endometriotic tissue might be involved in the development of this disorder [9, 10].

It has been well established that endometriosis patients show a dysfunctional immune system, where diverse immune cell types and inflammation-associated factors are increased in both peritoneal cavity and serum [11-13]. Nuclear factor kappa B (NF-κB) is a collection of master regulators of pro-inflammatory signals involved in diverse medical pathologies, such as autoinflammatory diseases and cancer [14, 15]. Previous studies have also shown that NF-κB was constitutively activated in endometriotic cells in endometriosis [16, 17], and suppression of NF-κB signaling pathway decreased the invasive and proliferative capabilities of endometriotic cells in endometriosis [18-20], emphasizing the crucial roles played by NF-κB in the pathogenesis of endometriosis.

Caspase recruitment domain family member 14 (CARD14) belongs to the CARD and membrane-associated guanylate kinase-like domain-containing protein (CARMA) family which act as a scaffolding protein to recruit B-cell lymphoma protein 10 (BCL10) and mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1) to assemble into signal-dependent CARD-BCL10-MALT1 complex, resulting in the activation of NF-κB signaling [21, 22]. Prior studies have shown that CARD14 mutations could activate NF-κB signaling pathway and cause pityriasis rubra pilaris and psoriasis [23-25]. Until now, it is largely unclear whether CARD14 mutations also exist in other human diseases. Considering the following facts that NF-κB signaling is frequently activated in endometriotic cells in endometriosis [16, 17], CARD14 mutations could activate the NF-κB signaling pathway [21,

Revised manuscript accepted for publication February 1, 2018

Table 1. — *The primer sequence for PCR amplification of the coding region and intron/exon boundaries of CARD14 gene.*

Exon	Forward primer (5'-3')	Reverse primer (5'-3')	Annealing temperature (°C)	Amplicon (base pairs)
2	taaaacggtgtcaccctg	tgaagacagaggcagaca	52	382
3	cttggtagctgggttctg	aggaactgcagcctggag	56	307
4	cccacctgcccacctattac	ggggcagggaagtgttagac	55	510
5	ctgtttccatcgcccttcct	ccaattttggggtctgggga	52	395
6	gggattctgcttgcctaggg	acagatgaacaggccgacag	50	332
7, 8	tccccgacccccttctaag	gctctgtcccactgtcacc	58	837
9	gtggtgctgacctggtaga	ggctcactttcgtcctga	55	285
10, 11	ctccagtcagttctcact	gaagttgagctctgcttct	60	690
12, 13	tgaccaagatctgtgaag	ccagcccagagtggatctg	57	712
14, 15	cgcctcagtgccctcagc	ggtctcaccacgcccacc	53	878
16, 17	ctgccctgctcacctggca	atcctggcctaaatgagt	50	692
18, 19	gcagggtaggctggctgg	aggtcacccaggtctcaggt	58	792
20	cctcagcctgtccggagg	acactccagaggcacctg	60	301
21	tcaaagcgaggccacctgt	cgtggtgagggttcaagg	52	483

25] and endometriotic lesions usually harbor somatic mutations in multiple genes [5-8]. The authors thus hypothesizes that the endometriotic lesions in endometriosis patients might possess CARD14 mutations. In the present study, they recruited a collection of 101 samples with ovarian endometriosis to test this hypothesis.

Materials and Methods

Only samples with more than 30% of endometrial tissues in the ectopic endometrial lesions were included in the present study. According to this criterion, the ectopic endometrial lesions and paired peripheral blood mononuclear cells were obtained from 101 samples with ovarian endometriosis from the Departments of Gynecology at Jiangxi Provincial Maternal and Child Health Hospital (Nanchang, China). All these ovarian endometriosis samples were cancer-free, as diagnosed by two experienced pathologists. This study was approved by the institutional review board of Jiangxi Provincial Maternal and Child Health Hospital and was performed according to the Declaration of Helsinki. All of these patients signed the informed consent prior to this study.

The following clinical data quantified as previously described [26] determined for the samples with ovarian endometriosis: age at the time of diagnosis, age of menarche, the serum hemoglobin, thyroid stimulating hormone (TSH), free triiodothyronine (FT3), free thyroxine (FT4), α-fetoprotein (AFP), carcinoembryonic antigen (CEA), cancer antigen 125 (CA125), and squamous cell carcinoma (SCC). In addition, white blood cell, lymphocyte cell, eosinophil granulocyte, mononuclear cell, neutrophil granulocyte, platelet count, and neutrophil granulocyte proportion were determined by an automated blood coagulation analyzer.

The genomic DNA of the ectopic endometrial lesions and paired peripheral blood mononuclear cells were isolated with tissue DNA and blood DNA kits, respectively. The isolated DNA was quantified by using a spectrophotometer. The PCR reactions were used to amplify the entire coding regions and the adjacent intron/exon boundaries of the CARD14 gene in the ectopic endometrial lesions with a set of primer pairs (Table 1). Each PCR reaction was carried out in a total volume of 30 μL containing 3 μL of 10 x PCR buffer, 0.2 μM dNTPs (2.5 mM each), 0.5 μM of each primer, 2.5 mM of $MgCl_2$, and 1.2U Taq DNA polymerase. The PCR reactions were carried out by using a thermal cycler . Followed by the purification of the PCR products, sequencing was performed using a sequencing kit and an automatic capillary DNA sequencer. For the detected mutations in the ectopic endometrial lesions, the paired peripheral blood mononuclear cells were sequenced to verify whether the identified mutations were somatic.

The authors adopted DeepView Swiss-PdbViewer 4.0 software to evaluate the potential protein structural change for the identified CARD14 mutation, it replaces the native amino acid with the mutant amino acid. A PDB structure of human CARD14 (5ubt.1.B) is available in the SWISS-MODEL Repository (https://swissmodel.expasy.org/repository/) in the ExPASy data- base (http://www.expasy.org). Based on this protein structure, human CARD14 wild-type and CARD14 p.V239A mutant proteins were modeled by select "show dots surface", "show backbone oxygen', "Use OpenGL Rendering" and "Sender in solid 3D" in the "displaying" option, and "by type" in the "Color" option in the toolbar of the DeepView Swiss-PdbViewer 4.0 software. The CARD14 native protein (239V) was modeled firstly and the CARD14 mutant protein (239A) was modeled by replacing alanine with valine in the 239th amino acid residue.

The Student's *t*-test was performed to compare the potential association between nominal variables referring to absence or presence of CARD14 mutations, and continuous variables were tested by Mann-Whitney method. All *p*-values were two-tailed and *p* values less than 0.05 were considered as statistical significance. Statistical processing of data was performed by using SPSS 18.0 software.

Results

A novel heterozygous missense CARD14 mutation, p.R470C (c.1408C>T), was identified in two out of 101 (2.0%) endometriotic lesions; this mutation was confirmed to be somatic by analyzing the paired peripheral blood mononuclear cells (Figure 1). This mutation was not found in ExAC (http://exac.broadinstitute.org/), 1000G (http://www.internationalgenome.org/) or COSMIC (http://cancer.sanger.ac.uk/cosmic) databases. The mutated samples were 30- and 31-years-old and both had a history of childbirth. No CARD14 mutations were detected in the remaining 99 samples with ovarian endometriosis.

The major clinical characteristics of the 101 samples with ovarian endometriosis are summarized in Table 2. The potential association of CARD14 mutation with these clinical features was evaluated by SPSS software. However, no as-

Table 2. — *Association of CARD14 mutations with clinical characteristics in 101 samples with ovarian endometriosis in the present study.*

Features	Total sample (n=101)	Wild type (n=99)	Wild type	Mutant type (n=2)	Mutant type	*p* value
Age (years)	101	99	32.55±7.40	2	30.50±0.71	0.55
Age of menarche (years)	101	99	13.65±1.32	2	15±1.41	0.17
Hemoglobin (g/l)	101	99	119.47±12.22	2	133±2.83	0.12
TSH (mIU/ml)	101	99	2.56±1.24	2	2.73±1.41	0.85
FT3 (pg/ml)	101	99	3.05±0.11	2	3.36±0.33	0.23
FT4 (ng/dl)	101	99	1.29±0.13	2	1.25±0.07	0.60
AFP (ng/ml)	101	99	2.49±1.81	2	3.39±0.92	0.48
CEA (ng/ml)	101	99	1.10±0.56	2	0.81±0.07	0.48
CA125 (μ/ml)	101	99	102.20±183.71	2	46.83±24.31	0.67
SCC (ng/ml)	101	99	1.44±1.03	2	1.89±1.73	0.55
White blood cell count ($x10^9$)	101	99	6.20±2.21	2	6.15±0.15	0.97
Lymphocyte cell count ($x10^9$)	101	99	1.90±0.54	2	1.40±0.17	0.19
Eosinophil granulocyte ($x10^9$)	101	99	0.12±0.11	2	0.07±0.06	0.57
Mononuclear cell count ($x10^9$)	101	99	0.46±0.19	2	0.35±0.03	0.41
Neutrophil cell count ($x10^9$)	101	99	3.78±2.09	2	4.28±0.01	0.74
Platelet ($x10^9$)	101	99	205.40±57.35	2	182.00±38.18	0.56
Neutrophil cell proportion (%)	101	99	58±9.80	2	69.70±1.70	0.095

AFP: α-fetoprotein; TSH: thyroid stimulating hormone; FT3: free triiodothyronine; FT4: free thyroxine; CEA: carcinoembryonic antigen; CA125: cancer antigen 125; SCC: squamous cell carcinoma.

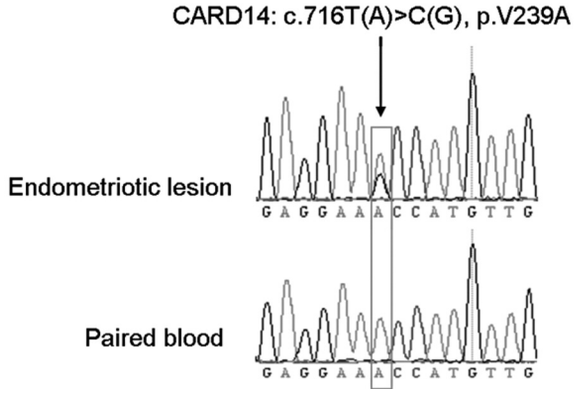

Figure 1. — The representative sequencing electropherograms of CARD14 mutations. The arrow refers to locations of the mutation.

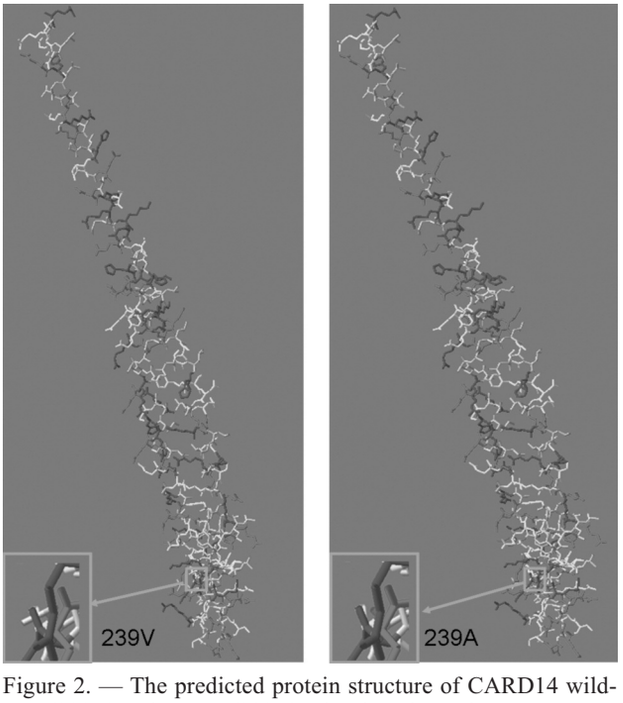

Figure 2. — The predicted protein structure of CARD14 wild-type (239V) and mutant (239A). The altered protein structures are labeled.

sociation was observed between these clinical features and CARD14 mutations.

The results of protein structural modeling showed that the newly identified CARD14 p.V239A mutation, exhibited structural changes (239A) when compared with the native CARD14 (239V) (Figure 2).

Discussion

Increased evidence has suggested that NF-κB signaling and inflammation play crucial roles in the pathogenesis of endometriosis, including promoting cell proliferation, invasion, and migration in endometriosis [19, 27, 28]. On the other hand, mutations in CARD14, one component of the CARD14-BCL10-MALT1 complex, could activate NF-κB signaling pathways and inflammation in pityriasis rubra pilaris and psoriasis [23, 25].

Previous studies have identified frequent CARD14 mutations in patients with pityriasis rubra pilaris and psoriasis [23-25]. In the present study, the authors identified a het-

erozygous missense somatic mutation, p.R470C (c.1408C>T), in two out of 101 (2.0%) endometriotic lesions by sequencing the entire coding regions and the adjacent intron/exon boundaries of the CARD14 gene for the first time. Database search showed that this mutation was not reported previously, neither found in ExAC nor 1000G databases; in addition, it was also not reported in the COSMIC database. Considering the facts that CARD14 p.R470C (c.1408C>T) mutation occurred in two individuals and this mutation was predicted to alter the protein secondary structure, the authors thus speculated that this mutation might be deleterious. Of note, two recent studies based on large-scale DNA sequencing technology have failed to identify any CARD14 mutations in either 16 endometriotic lesions of ovarian endometriosis [8] or 27 endometriotic lesions of deeply infiltrating endometriosis [7]. For the differential CARD14 mutation frequency between the present authors' and the prior studies, they speculated that the relative small sample size analyzed in the prior studies might be a main reason; alternatively, another explanation for the differential CARD14 mutation might be that the molecular pathogenic mechanisms between ovarian endometriosis and deeply infiltrating endometriosis is different.

Prior studies have shown that disease-associated CARD14 mutations could activate NF-κB and enhance pro-inflammatory signals and then promote the development of psoriasis and pityriasis rubra pilaris [29, 30]. Nevertheless, there were no substantial differences in inflammation-associated clinical characteristics between samples with or without CARD14 mutations in this study, including white blood cell, lymphocyte cell, neutrophil granulocyte, eosinophil granulocyte, mononuclear cell, platelet count, and neutrophil granulocyte proportion, implicating that the inflammation signals in ovarian endometriosis might be caused by many factors, and the detailed process might be more complex than what was thought. In addition, no association between CARD14 mutations and any other available clinical features was observed in this sample cohort, including age at diagnosis, age of menarche, hemoglobin, TSH, FT3, FT4, AFP, CEA, CA125, and SCC. These results implicated that CARD14 mutations might not be involved in the production or turnover processes of these factors. It should be mentioned that, due to the limited sample size with CARD14 mutation (n=2) in this sample cohort, this conclusion should be treated cautiously.

Conclusion

The authors identified a novel CARD14 somatic mutation in 2/101 endometriotic lesions with ovarian endometriosis for the first time, and this mutation might be a damaging mutation and thus might play an active role in the pathogenesis of ovarian endometriosis.

Acknowledgements

The authors thank the sample donors involved in this study. This study was supported by grants from Jiangxi Provincial Science and Technology Plan Project (No. 20143ACG70016), Jiangxi Provincial Natural Science Foundation (20161ACB21021) and GanPo Talent 555 Project of Jiangxi Province (for Huang OP).

References

[1] Miravet-Valenciano J., Ruiz-Alonso M., Gómez E., Garcia-Velasco J.A.: "Endometrial receptivity in eutopic endometrium in patients with endometriosis: it is not affected, and let me show you why". *Fertil. Steril.,* 2017, *108*, 28.

[2] Greene A.D., Lang S.A., Kendziorski J.A., Sroga-Rios J.M., Herzog T.J., Burns K.A.: "Endometriosis: where are we and where are we going?". *Reproduction.,* 2016, *152*, R63.

[3] Koga K., Takamura M., Fujii T., Osuga Y.: "Prevention of the recurrence of symptom and lesions after conservative surgery for endometriosis". *Fertil. Steril.,* 2015, *104*, 793.

[4] Laganà A.S., Vitale S.G., Trovato M.A., Palmara V.I., Rapisarda A.M., Granese R., *et al.:* "Full-thickness excision versus shaving by laparoscopy for tntestinal deep infiltrating endometriosis: rationale and potential treatment options". *Biomed. Res. Int.,* 2016, *2016*, 3617179.

[5] Giudice LC.: "Clinical practice. Endometriosis". *N. Engl. J. Med.,* 2010, 362(25), 2389-2398.

[6] Brown J., Farquhar C.: "Endometriosis: an overview of Cochrane Reviews". *Cochrane. Database. Syst. Rev.,* 2014, *3*, CD009590.

[7] Anglesio M.S., Papadopoulos N., Ayhan A., Nazeran T.M., Noë M., Horlings H.M., *et al.*: "Cancer-associated mutations in endometriosis without cancer". *N. Engl. J. Med.,* 2017, *376*, 1835.

[8] Li X., Zhang Y., Zhao L., Wang L., Wu Z., Mei Q., *et al.:* "Whole-exome sequencing of endometriosis identifies frequent alterations in genes involved in cell adhesion and chromatin-remodeling complexes". *Hum. Mol. Genet.,* 2014, *23*, 6008.

[9] Ahn S.H., Monsanto S.P., Miller C., Singh S.S., Thomas R., Tayade C.: "Pathophysiology and immune dysfunction in endometriosis". *Biomed. Res. Int.,* 2015, *2015*, 795976.

[10] Miller J.E., Ahn S.H., Monsanto S.P., Khalaj K., Koti M., Tayade C.: "Implications of immune dysfunction on endometriosis associated infertility". *Oncotarget*., 2017, *8*, 7138.

[11] Jeung I., Cheon K., Kim M.R.: "Decreased cytotoxicity of peripheral and peritoneal natural killer cell in endometriosis". *Biomed. Res. Int.,* 2016, 2016, 2916070.

[12] Schulke L., Berbic M., Manconi F., Tokushige N., Markham R., Fraser I.S.: "Dendritic cell populations in the eutopic and ectopic endometrium of women with endometriosis". *Hum. Reprod.,* 2009, 24(7), 1695-1703.

[13] Harada T., Iwabe T., Terakawa N.: "Role of cytokines in endometriosis". *Fertil. Steril.,* 2001, *76*, 1-10.

[14] Zilberman-Rudenko J., Shawver L.M., Wessel A.W., Luo Y., Pelletier M., Tsai WL., *et al.*: "Recruitment of A20 by the C-terminal domain of NEMO suppresses NF-κB activation and autoinflammatory disease". *Proc. Natl. Acad. Sci. U. S. A.,* 2016, *113*, 1612.

[15] Hoesel B., Schmid J.A.: "The complexity of NF-κB signaling in inflammation and cancer". *Mol. Cancer.,* 2013, *12*, 86.

[16] Kim S.H., Ihm H.J., Oh Y.S., Chae H.D., Kim C.H., Kang B.M.: "Increased nuclear expression of nuclear factor kappa-B p65 subunit in the eutopic endometrium and ovarian endometrioma of women with advanced stage endometriosis". *Am. J. Reprod. Immunol.,* 2013, *70*, 497.

[17] González-Ramos R., Donnez J., Defrère S., Leclercq I., Squifflet J., Lousse J.C., Van Langendonckt A.: "Nuclear factor-kappa B is constitutively activated in peritoneal endometriosis". *Mol. Hum. Re-*

prod., 2007, *13*, 503.
[18] Grund E.M., Kagan D., Tran C.A., Zeitvogel A., Starzinski-Powitz A., Nataraja S., Palmer S.S.: "Tumor necrosis factor-alpha regulates inflammatory and mesenchymal responses via mitogen-activated protein kinase kinase, p38, and nuclear factor kappaB in human endometriotic epithelial cells". *Mol. Pharmacol.,* 2008, *73*, 1394.
[19] Dai L., Gu L., Di W.: "MiR-199a attenuates endometrial stromal cell invasiveness through suppression of the IKKβ/NF-κB pathway and reduced interleukin-8 expression". *Mol. Hum. Reprod.,* 2012, *18*, 136.
[20] González-Ramos R., Defrère S., Devoto L.: "Nuclear factor-kappaB: a main regulator of inflammation and cell survival in endometriosis pathophysiology". *Fertil. Steril.,* 2012, *98*, 520.
[21] Bertin J., Wang L., Guo Y., Jacobson M.D., Poyet J.L., Srinivasula S.M., *et al.:* "CARD11 and CARD14 are novel caspase recruitment domain (CARD)/membrane-associated guanylate kinase (MAGUK) family members that interact with BCL10 and activate NF-kappa B". *J. Biol. Chem.,* 2001, *276*, 11877.
[22] Sanada T., Kim M., Mimuro H., Suzuki M., Ogawa M., Oyama A., *et al.:* "The Shigella flexneri effector OspI deamidates UBC13 to dampen the inflammatory response". *Nature.,* 2012, *483*, 623.
[23] Fuchs-Telem D., Sarig O., van Steensel M.A., Isakov O., Israeli S., Nousbeck J., *et al.:* "Familial pityriasis rubra pilaris is caused by mutations in CARD14". *Am. J. Hum. Genet.,* 2012, 91(1), 163-170.
[24] Tang H., Jin X., Li Y., Jiang H., Tang X., Yang X., *et al.:* "A large-scale screen for coding variants predisposing to psoriasis". *Nat. Genet.,* 2014, 46(1), 45-50.
[25] Jordan C.T., Cao L., Roberson E.D., Duan S., Helms C.A., Nair R.P., *et al.:* "Rare and common variants in CARD14, encoding an epidermal regulator of NF-kappaB, in psoriasis". *Am. J. Hum. Genet.,* 2012, *90*, 796.
[26] Wu J., Zou Y., Luo Y., Guo J.B., Liu F.Y., Zhou J.Y., *et al:,* "Prevalence and clinical significance of mediator complex subunit 12 mutations in 362 Han Chinese samples with uterine leiomyoma". *Oncol. Lett.,* 2017, *14*, 47.
[27] Soares S.R., Martínez-Varea A., Hidalgo-Mora J.J., Pellicer A.: "Pharmacologic therapies in endometriosis: a systematic review". *Fertil. Steril.,* 2012, *98*, 529.
[28] Wang Y., Lin M., Weng H., Wang X., Yang L., Liu F.: "ENMD-1068, a protease-activated receptor 2 antagonist, inhibits the development of endometriosis in a mouse model". *Am. J. Obstet. Gynecol.,* 2014, *210*, 531.e1.
[29] Takeichi T., Sugiura K., Nomura T., Sakamoto T., Ogawa Y., Oiso N., *et al.:* "Pityriasis rubra pilaris type V as an autoinflammatory disease by CARD14 mutations". *JAMA. Dermatol.,* 2017, *153*, 66.
[30] Jordan C.T., Cao L., Roberson E.D., Pierson K.C., Yang C.F., Joyce C.E., *et al.:* "PSORS2 is due to mutations in CARD14". *Am. J. Hum. Genet.,* 2012, *90*, 784.

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