Familial Cancer (2011) 10:127–132
DOI 10.1007/s10689-010-9393-y

Identification of a large rearrangement in *CYLD* as a cause of familial cylindromatosis

Ans M. W. van den Ouweland · Peter Elfferich · Roy Lamping · Raoul van de Graaf · Monique M. van Veghel-Plandsoen · S. M. Franken · A. C. Houweling

Published online: 23 October 2010

Abstract Pathogenic mutations in *CYLD* can be identified in patients affected with Brooke-Spiegler syndrome, (Familial) Cylindromatosis or multiple familial trichoepithelioma. To date, only technologies which are able to identify small point mutations in *CYLD*, such as sequence and WAVE analysis, were used. Here we describe the identification of a larger rearrangement identified by Quantitative PCR analysis of *CYLD*, indicating that a combination of these technologies is necessary when searching for pathogenic mutations in *CYLD*.

Keywords *CYLD* · Familial cylindromatosis · Large rearrangement · Mutation analysis · Q-PCR analysis

Introduction

Brooke-Spiegler syndrome (BSS; OMIM #60541) is an autosomal dominant disorder characterised by cylindromas, trichoepitheliomas and spiradenomas, especially in the head and neck region. Due to the clinical overlap with (familial) Cylindromatosis (FC; OMIM #132700) and multiple Familial Trichoepithelioma (MFT; OMIM #601606), these disorders are considered to represent a single disease entity [1–3].

Linkage analysis was used to define the familial cylindromatosis region, resulting in the identification of *CYLD* [4–6]. Germline mutations in *CYLD* have been identified in BSS, FC and MFT patients, in agreement with the clinical findings indicating that these disorders have a common genetic basis [6–11]. *CYLD* is encoded by 20 exons of which exon 4 contains the ATG start codon [6]. The CYLD protein is a deubiquitinating enzyme regulating cell signalling via several pathways, including the NF-κB and JNK pathways [12–14].

Recently, a nice overview was published by Blake and Toro where they described 51 distinct germline mutations in *CYLD* in 73 families with BSS, FC and MFT [7]. All types of mutations were identified: frameshift (41%), nonsense (35%), missense (14%) and putative splice site (10%). No large rearrangements were identified, probably because all techniques used were not suitable for identifying this type of mutation. Loss of heterozygosity is shown in cylindromas and trichoepitheliomas, indicating that *CYLD* acts as a tumour suppressor gene [6, 8, 15–17]. This observation is in agreement with the function of the CYLD protein in regulating several pathways in cell signalling. In most tumour suppressor genes, e.g. *BRCA1*, *NF1*, *TSC2*, large rearrangements are identified as pathogenic germline mutations [18–20]. Therefore we hypothesized that larger rearrangements in *CYLD* could be identified in patients with BSS, FC and MFT.

Here we describe the identification of a large rearrangement in *CYLD* in a patient with FC using Quantitative (Q)-PCR, indicating that a quantitative test should be performed on DNA samples of patients if no mutation was identified by techniques which can only identify small sequence changes, such as sequence or WAVE analysis.

A. M. W. van den Ouweland (✉) · P. Elfferich · R. Lamping · R. van de Graaf · M. M. van Veghel-Plandsoen
Department of Clinical Genetics, Erasmus Medical Center, P.O. Box 2040, 3000 CA Rotterdam, The Netherlands
e-mail: a.vandenouweland@erasmusmc.nl

S. M. Franken
Department of Dermatology, VU University Medical Center, Amsterdam, The Netherlands

A. C. Houweling
Department of Clinical Genetics, VU University Medical Center, Amsterdam, The Netherlands

Materials and methods

Patient samples

In our diagnostic setting we received 13 samples from patients with Brooke-Spiegler syndrome (BSS, n = 2), (Familial) Cylindromatosis [(F)C; n = 7] or Trichoepithelioma (T; n = 2). Of the remaining two patients the indication for testing *CYLD* was not provided by the applicant (Table 1). Clinical details were obtained only from the patient described in this paper.

Mutation analysis

Extraction of DNA from peripheral blood cells was performed according to standard techniques. Mutation analysis of the coding exons 4–20 and exon/intron boundaries of *CYLD* was performed by sequence analysis (primers available on request) using an automated sequencer (ABI 3730XL, Applied Biosystems, Foster City, CA, USA). Data were analysed using SeqScape software (version 2.6; Applied Biosystems). If a sequence change could not be classified as a pathogenic mutation, the sequence change was analysed using Alamut software (Mutation Interpretation Software; version 1.5 May 2009; Interactive Biosoftware, Rouen, France).

In case no pathogenic mutation or an unclassified variant was identified, Quantitative (Q)-PCR analysis was performed to search for large rearrangements.

Quantitative-PCR, Long-range-PCR and sequence analysis of breakpoints

Real-time Q-PCR was performed using Fam-labelled Taqman assays. Oligonucleotides were designed for all (non) coding exons. If the CG content was too high (exon 1) or too low (exons 14–17), a Taqman assay was designed using oligonucleotides in the promoter region (instead of exon 1) and in the intronic regions between exons 14 and 17 as close as possible to the exons (Table 2). Oligonucleotides were designed with Primer Express 2.0.0 (Applied Biosystems). Primer specificity was checked by performing BLAST analysis. Taqman probes were synthesised with a melting temperature (*Tm*) 8–10°C higher than the primers by incorporating Locked Nucleic Acid (LNA) monomers in the probe. *Tm* values for the LNA probes were calculated using the Exiqon website (http://lna-tm.com/). The LNA-based Taqman assays were manufactured by Eurogentec (Maastricht, The Netherlands). Since LNA probes show a high thermal stability and are resistant to exo- and endonuclease activity [21], we prefer the use of these probes in the Taqman assay.

Gene dosage alterations were detected on an ABI7500 Real time PCR system (Applied Biosystems) by performing a relative quantification run. Real time PCR reactions were performed in a total volume of 25 µl, containing 20 ng DNA, 1 × qPCR mastermix Plus–low ROX (Eurogentec: RT-QP2x-03-WOULR), 1 × RNAse P (endogenous control; Applied Biosystems), 30 µM forward and reverse primers and 10 µM probe. PCR conditions were as follows: an initial 2 min incubation at 50°C, followed by 95°C for 10 min and then 40 cycles of 95°C for 15 s and 60°C for 1 min. All samples were analysed in triplicate and compared to a normal control sample.

LR-PCR was performed with the Expand Long Template PCR System (Roche Applied Science, Indianapolis, IN, USA). The product obtained by LR-PCR was sequenced using an automated sequencer (ABI 3730XL). Nomenclature of the deletion was according to the recommendations of the Human Genome Variation Society, using reference sequence NM_015247_2. After characterisation of the breakpoints in patient 37999, a deletion-specific PCR

Table 1 Overview of patients analysed for mutations in *CYLD*

ID number	Gender	Indication	Mutation		Exon
41356	F	ND	c.1112C>A	p.Ser371X	9
14872	F	T	c.1682T>A	p.Leu561X	11
17514	F	C	c.2065_2066delCT	p.Leu689fs	15
17559	F	FC	c.2068_2069delTTinsC	p.Phe690fs	15
35680	F	C	c.2146C>A*	p.Gln716K	16
22748	F	C	c.2272C T	p.Arg758X	17
41440	F	BSS	c.2350+5G>A*	p.?	17
42008	F	FC	c.2655G>A	p.Trp885X	19
11468	F	FC	c.2662_2664delTTT*	p.Phe888del	19
37999	F	FC	c.2686+60_*3340del5362	p.?	20
28597	F	BSS		normaal	
30945	F	T		normaal	
32565	M	ND		normaal	

BSS Brooke-Spiegler syndrome
C Cylindromatosis
FC Familial cylindromatosis
ND No data
T Trichoepithelioma
* Unclassified variant

Table 2 Overview of oligonucleotides used for Q-PCR analysis and deletion-specific breakpoint PCR

Location	Taqman oligonucleotides	cDNA numbering 5′–3′
Promoter	ggctcagcgtggttgtgact	c.−415 − 516
	tctttggcgctttcattcagt	c.−415 − 458
	cctcapgtcacgcagc	c.−415 − 495
Exon 2	tttctagggtgaggatggttctaca	c.−332
	agggcgcacctttcaactaag	c.−271
	agclaclcgpagtt	c.−306
Intron 3	tgtcttactgttccctaggccttt	c.−124 + 398
	cccaacatcaatccacattcac	c.−124 + 462
	cttaaapglapaapctg	c.−124 + 423
Exon 4	cgtgggctgtcctgtgaaa	c.378
	agcgtacaactccaggaaattttt	c.442
	tacegltpapatltgg	c.398
Exon 5	ggagaaacaatagaatctggaacagtt	c.721
	ccacaccaacaaaatatcctaagct	c.802
	tgtgatpttttglcapgaa	c.754
Exon 6	ggcaactgggatggaagatt	c.820
	ttgtactttcaacacacgcaaaact	c.883
	atgpaptpcegltttt	c.842
Exon 7	ctcaaatccactgtgggtgatatc	c.914 − 42
	tccaacacgacacttaggagtca	c.922 + 26
	tttttpctgalacalagc	c.914 − 17
Exon 8	agagtgtgacgcaggaaagga	c.923
	tgtccccaacacctcttgaca	c.985
	cctcclaaalttpcctt	c.946
Intron 9	tgtgcagtgaaggtgcatga	c.1138 + 537
	cccctaagaccctgagaaaaatc	c.1138 + 602
	ctgtpapaatalaclaaag	c.1138 + 559
Exon 10	tggccacagtccactttctct	c.1299
	cgggtgcagtgtttagctctt	c.1360
	tcapcclagtltptaatg	c.1321
Exon 11	gctgtacggatggaaccttca	c.1535
	cacaaacagcgccttcttca	c.1602
	tcggtatttlalctgtgcc	c.1563
Exon 12	cttggagataatgattgggaagaag	c.1749
	gaataaggttgagtctaagtaacaagaattgt	c.1824
	aagglatclagpgtc	c.1775
Exon 13	tcttttgcagcttatttgcttttagt	c.1827 − 10
	catcgttcttttctttgggtctaagt	c.1888
	ctgttltpgacaltgtg	c.1844
Intron 14	aaagattcaccacctgactttgaat	c.2042 − 399
	ttcctgcaagcctctgaacatt	c.2042 − 326
	tgtlapcaglataltgta	c.2042 − 373
Intron 15	agcgccttcattttagaaatgaa	c.2109 − 456
	tgctggatgtgacagaccctta	c.2109 − 390
	agttlgazctapaapgtc	c.2109 − 432
Intron 16	tcaagattattgaactctgtgacctctag	c.2242 − 243
	caagtcttcaagtggctcatgatc	c.2242 − 173
	tgctpgtgtpcccc	c.2242 − 213
Exon 18	ctcacatttcagctcccagaca	c.2351 − 12
	gcattctctacactcatacattgcaa	c.2406
	tgccgpatatgtpgagg	c.2362
Exon 19	cccagtgtcacttcccaaaga	c.2508
	aagggatgcagccgtgtct	c.2566
	ttacclgactpggactg	c.2530
Exon 20	aagatgtctctggaagacctgcat	c.2749
	ttcgtgcacagccttgga	c.2809
	ccttpgactccapgaga	c.2774
Primers for deletion PCR		
c.2686+60_*3340del5362		
Forward	cccagtgtcacttcccaaaga	c.2508
Reversed	tcgtccctactctgccactt	c.*3563
Internal	cctcaagccccttaaacctc	c.2686 + 715

cDNA numbering is according to reference sequence NM_015247_2. In the oligonucleotides used in the Taqman assay, the following abbreviations are used for the LNA incorporations: E, A-LNA; L, C-LNA; P, G-LNA and Z, T-LNA

analysis was designed. Three primers (Table 2) were used: a common primer, a primer specific for the deletion and a primer specific for the wildtype allele. The primers were designed in such a way that the PCR product from the allele carrying the deletion was shorter than the product from the wildtype allele. The PCR conditions were: an initial 2 min incubation at 94°C, followed by 30 cycles of 94°C for 60 s, 60°C for 30 s and 72°C for 90 s and completed by 72°C for 10 min.

Results

Mutation analysis

Using sequence analysis of all coding exons and exon intron boundaries of *CYLD,* a pathogenic mutation was identified in 6 out of 13 patients of our diagnostic cohort (Table 1). Of these mutations, c.1112C>A (p.Ser371X) and c.2272C>T (p.Arg758X) were previously described. The c.2272C>T (p.Arg758X) mutation was found in two families with different haplotypes, indicating that this mutation is a recurrent mutation in *CYLD* [6]. In three patients, an unclassified variant was identified: c.2146C>A (p.Gln716K), c.2350+5G>A and c.2662_2664delTTT. One of these variants, c.2350+5G>A was previously described, but no formal proof that this sequence change is a pathogenic mutation was provided [6]. Three splice site

prediction programs of Alamut (SpliceSiteFinder-like, MaxEntScan and NNSPLICE) showed that it is very likely that the sequence change c.2350+5G>A will have an effect on RNA splicing (data not shown). The fourth program, GeneSplicer, did not recognise the wildtype donor site and therefore no conclusion could be drawn from this program. Since we did not receive relevant family members to analyse the presence/absence of c.2350+5G>A, or a new sample of the index patient to perform RNA analysis, we were not able to prove that c.2350+5G>A is a pathogenic splice site mutation. No DNA of relevant relatives of the patients with the unclassified variants c.2146C>A (p.Gln716K) and c.2662_2664delTTT was received to obtain further information with respect to the pathogenic character of these sequence changes.

No pathogenic mutation or unclassified variant was identified in four patients.

Identification and characterisation of the large rearrangement

To identify larger rearrangements, a quantitative technique must be applied. We developed Q-PCR analyses for all exons/introns of *CYLD* and DNA of all patients without a pathogenic mutation, including the patients carrying an unclassified variant, was analysed. Q-PCR analysis of DNA of patient 37999 showed a pattern in agreement with a deletion of exon 20 (Fig. 1). No abnormal patterns were identified in DNA of the other 6 patients, indicating that no large rearrangements are present in these patients (data of the other exons: not shown).

To characterise the breakpoints, LR-PCR was performed and the PCR product was sequenced. The deletion started 60 nucleotides after exon 19 and extended 3340 nucleotides after the translation stopcodon of *CYLD*. In total 5362 nucleotides were deleted (c.2686+60_*3340del5362; Fig. 2a and 2b). No (direct) repeat structures were present in the vicinity of the breakpoint which could explain the nature of the deletion. A deletion-specific PCR was developed, showing not only the wildtype allele of 913 basepairs, but also the mutant specific fragment of 462 basepairs (Fig. 2c). This analysis confirmed the presence of the pathogenic mutation in two independent DNA samples of the patient and can be used for further family analysis.

Clinical details of patient 37999

The patient, a 47-year old female, was referred because of the presence of multiple lenticular, smooth papules on the forehead and the scalp (Fig. 3). The lesions were reported to be present for 10–15 years. Similar lesions were

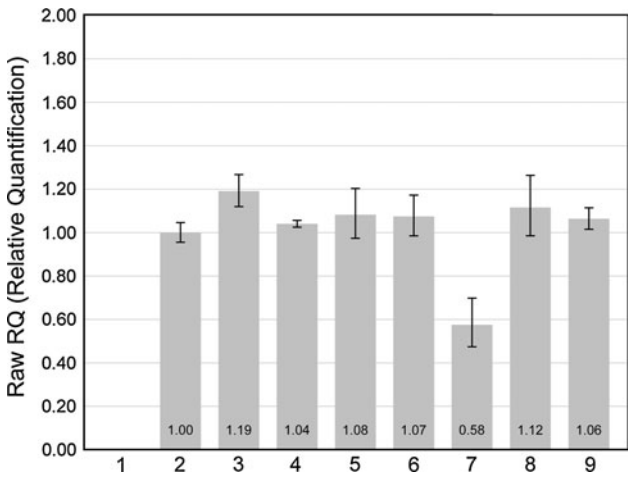

Fig. 1 Q-PCR result of exon 20 of patients with no pathogenic mutation or with an unclassified variant after sequence analysis of *CYLD*. *Lane 1* no DNA; *lane 2* negative control used as calibrator; *lane 3* patient 28597; *lane 4* patient 30945; *lane 5* patient 32565; *lane 6* patient 35680; *lane 7* patient 37999; *lane 8* patient 41440 and *lane 9* patient 11468. *Bars* represent Relative Quantification (RQ) calculated by $2^{-\Delta\Delta CT}$. On *top of the bars*, the standard error of the mean RQ value is displayed. *Inside the bars* the calculated RQ value is given of a triplicate measurement

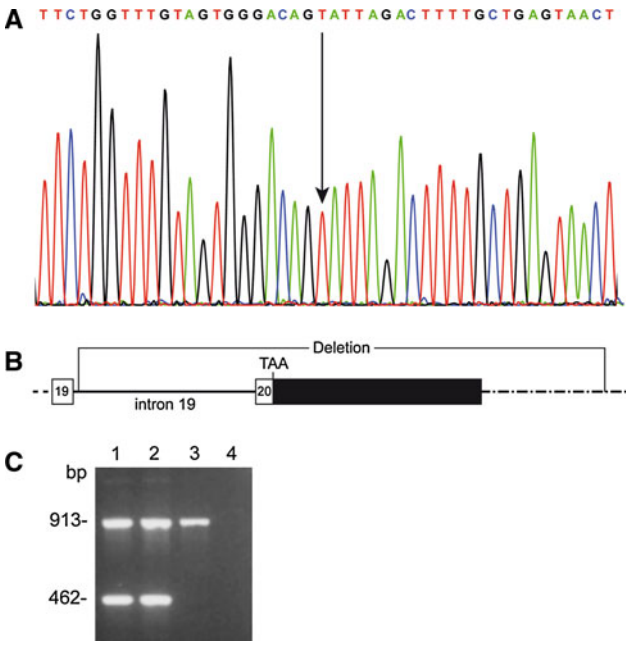

Fig. 2 Characterization of the breakpoint (c.2686+60_*3340del5362) of patient 37999. **a** Result of sequence analysis of the long-range PCR product. The arrow indicates the last nucleotide of intron 19 that is still present. **b** Schematic overview of the deletion present in patient 37999. Exon 19 and the coding part of exon 20 of *CYLD* are represented by open boxes and the noncoding part of exon 20 by a black box. The intergenic region is given by *dot with broken line* **c** Agarose gel electrophoresis of the deletion-specific PCR product. *Lane 1* and *2*, two independent DNA samples of patient 37999; *lane 3*, negative control DNA and *lane 4*, no DNA. The wildtype fragment is 913 bp in length and the mutant fragment 462 bp

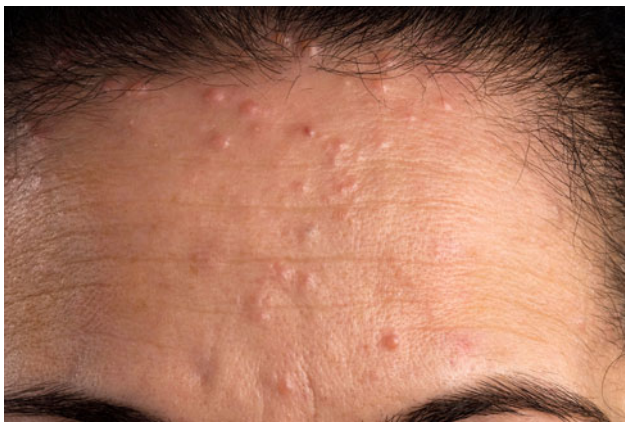

Fig. 3 Multiple lenticular, smooth papules on the forehead of patient 37999

confirmed to be present in her sister, and were reported by the patient to be present in her father, grandfather and her son. No other clinical features were present.

Discussion

Our diagnostic cohort of patients with a pathogenic mutation in *CYLD* comprised the clinical phenotypes resembling Brooke-Spiegler syndrome (BSS), (Familial) Cylindromatosis (FC) and (Multiple Familial) Trichoepithelioma (MFT). One BSS patient was heterozygous for the unclassified variant c.2350+5G>A. This sequence change was previously described as being a pathogenic mutation, but there was no formal proof for this conclusion [6]. *In silico* analysis showed that the sequence change c.2350+5G>A might have an effect on RNA splicing and therefore might be a pathogenic mutation. No pathogenic mutation was identified in the other BSS patient. Of the seven (F)C patients, five carried a pathogenic mutation, whereas the two other patients were carrier of an unclassified variant. In one of two MFT patients a pathogenic mutation was identified. In one of the two patients without information with respect to the indication for testing, a pathogenic mutation could be identified. Using a combination of sequence and Q-PCR analyses, 7 pathogenic and 3 unclassified variants in 13 patients (77%) were identified, comparable with the overall detection rate of 83% previously described [7]. All missense mutations described to date were located within the USP (Ubiquitin Specific Protease) domain (amino acids 583–956) [7, 22]. Therefore it is very likely that the unclassified variants c.2146C>A (p.Gln716K) and c.2662_2664delTTT (p.Phe888del) identified in our cohort, were also pathogenic mutations. In that case, a pathogenic mutation was identified in all our (F)C patients. This was comparable with the findings of Saggar et al., who identified pathogenic mutations in 100% (3/3) and 44% (4/9) of their FC and MFT patients, respectively, [9]. In agreement with all previously identified sequence change in *CYLD* [7], the localisation of the abnormalities described in this paper were in exons 9–20. In patient 37999 a large genomic rearrangement was identified using a quantitative analysis. This deletion (c.2686+60_*3340del5362) encompassed almost complete intron 19 and extended in the 3′ UTR of *CYLD*. If the allele carrying the deletion will lead to a stable mRNA, the mutant RNA will encode a protein lacking amino acids 896–956, which are part of the USP domain (aa 583–956). The mutant CYLD protein will lack a part of its functional domain and this explains the clinical phenotype of the patient. The frequency of this type of rearrangments in *CYLD* in our cohort is about 10% and is comparable with the frequencies observed in other genetic disorders such as Neurofibromatosis type 1 and Tuberous sclerosis complex [18, 19].

Since there is intra- en interfamilial variability in the clinical expression, it will be very hard to get a genotype-phenotype correlation. Our patient with a large rearrangement in *CYLD* was not severely affected. This might be due to the fact that the large deletion only encompassed *CYLD* and no additional gene(s). It had been suggested that there is a higher incidence of BSS, (F)C or MFT in females [23], most likely as a result of reduced penetrance in males [24]. In our cohort, 12 out of 13 patients were females (Table 1), in agreement with this hypothesis.

To our knowledge, this is the first large germline deletion identified in *CYLD*, indicating that screening for this type of mutations in a diagnostic setting is recommended in patients with Brooke-Spiegler syndrome, (familial) Cylindromatosis or multiple Familial Trichoepithelioma.

References

1. Welch JP, Wells RS, Kerr CB (1968) Ancell-Spiegler cylindromas (turban tumours), Brooke-Fordyce Trichoepitheliomas: evidence for a single genetic entity. J Med Genet 5(1):29–35
2. Young AL, Kellermayer R, Szigeti R, Teszas A, Azmi S, Celebi JT (2006) CYLD mutations underlie Brooke-Spiegler, familial cylindromatosis, multiple familial trichoepithelioma syndromes. Clin Genet 70(3):246–249
3. Oranje AP, Halley D, den Hollander JC et al (2008) Multiple familial trichoepithelioma, familial cylindroma: one cause!. J Eur Acad Dermatol Venereol 22(11):1395–1396
4. Biggs PJ, Wooster R, Ford D et al (1995) Familial cylindromatosis (turban tumour syndrome) gene localised to chromosome 16q12–q13: evidence for its role as a tumour suppressor gene. Nat Genet 11(4):441–443
5. Takahashi M, Rapley E, Biggs PJ et al (2000) Linkage and LOH studies in 19 cylindromatosis families show no evidence of

genetic heterogeneity, refine the CYLD locus on chromosome 16q12–q13. Hum Genet 106(1):58–65
6. Bignell GR, Warren W, Seal S et al (2000) Identification of the familial cylindromatosis tumour-suppressor gene. Nat Genet 25(2):160–165
7. Blake PW, Toro JR (2009) Update of cylindromatosis gene (CYLD) mutations in Brooke-Spiegler syndrome: novel insights into the role of deubiquitination in cell signaling. Hum Mutat 30(7):1025–1036
8. Kazakov DV, Thoma-Uszynski S, Vanecek T, Kacerovska D, Grossmann P, Michal M (2009) A case of Brooke-Spiegler syndrome with a novel germline deep intronic mutation in the CYLD gene leading to intronic exonization, diverse somatic mutations, unusual histology. Am J Dermatopathol 31(7):664–673
9. Saggar S, Chernoff KA, Lodha S et al (2008) CYLD mutations in familial skin appendage tumours. J Med Genet 45(5):298–302
10. Zhang XJ, Liang YH, He PP et al (2004) Identification of the cylindromatosis tumor-suppressor gene responsible for multiple familial trichoepithelioma. J Invest Dermatol 122(3):658–664
11. Zhang G, Huang Y, Yan K et al (2006) Diverse phenotype of Brooke-Spiegler syndrome associated with a nonsense mutation in the CYLD tumor suppressor gene. Exp Dermatol 15(12): 966–970
12. Brummelkamp TR, Nijman SM, Dirac AM, Bernards R (2003) Loss of the cylindromatosis tumour suppressor inhibits apoptosis by activating NF-kappaB. Nature 424(6950):797–801
13. Trompouki E, Hatzivassiliou E, Tsichritzis T, Farmer H, Ashworth A, Mosialos G (2003) CYLD is a deubiquitinating enzyme that negatively regulates NF-kappaB activation by TNFR family members. Nature 424(6950):793–796
14. Xue L, Igaki T, Kuranaga E, Kanda H, Miura M, Xu T (2007) Tumor suppressor CYLD regulates JNK-induced cell death in Drosophila. Dev Cell 13(3):446–454
15. Biggs PJ, Chapman P, Lakhani SR, Burn J, Stratton MR (1996) The cylindromatosis gene (cyld1) on chromosome 16q may be the only tumour suppressor gene involved in the development of cylindromas. Oncogene 12(6):1375–1377
16. Thomson SA, Rasmussen SA, Zhang J, Wallace MR (1999) A new hereditary cylindromatosis family associated with CYLD1 on chromosome 16. Hum Genet 105(1–2):171–173
17. Leonard N, Chaggar R, Jones C, Takahashi M, Nikitopoulou A, Lakhani SR (2001) Loss of heterozygosity at cylindromatosis gene locus, CYLD, in sporadic skin adnexal tumours. J Clin Pathol 54(9):689–692
18. De Luca A, Bottillo I, Dasdia MC et al (2007) Deletions of NF1 gene, exons detected by multiplex ligation-dependent probe amplification. J Med Genet 44(12):800–808
19. Kozlowski P, Roberts P, Dabora S et al (2007) Identification of 54 large deletions/duplications in TSC1, TSC2 using MLPA, genotype-phenotype correlations. Hum Genet 121(3–4):389–400
20. van den Ouweland AM, Dinjens WN, Dorssers LC et al (2009) Deletion of exons 1a–2 of BRCA1: a rather frequent pathogenic abnormality. Genet Test Mol Biomarkers 13(3):399–406
21. Ugozzoli LA, Latorra D, Puckett R, Arar K, Hamby K (2004) Real-time genotyping with oligonucleotide probes containing locked nucleic acids. Anal Biochem 324(1):143–152
22. Komander D, Lord CJ, Scheel H et al (2008) The structure of the CYLD USP domain explains its specificity for Lys63-linked polyubiquitin and reveals a B box module. Mol cell 29(4):451–464
23. van Balkom ID, Hennekam RC (1994) Dermal eccrine cylindromatosis. J Med Genet 31(4):321–324
24. Anderson DE, Howell JB (1976) Epitheliomaadenoides cysticum: genetic update. Br J Dermatol 95(3):225–232