Novel *TMC8* splice site mutation in epidermodysplasia verruciformis and review of HPV infections in patients with the disease

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Abstract

Background: Epidermodysplasia verruciformis (EV) is a genodermatosis leading to infections with cutaneous HPV, persistent plane warts and a high rate of non-melanoma skin cancer (NMSC). Biallelic loss-of-function mutations in *TMC6* and *TMC8* are known to be causative.

Objective: The aim of this study was to report EV-causing mutations in four patients with EV and to give an overview of all described EV patients.

Patients and methods: We investigated four patients with classical features of EV from two families. All patients were affected by plane warts with typical EV histology since early childhood and β-HPVs were detected on their skin. One patient had recurring cutaneous squamous cell carcinomas (cSCC) and carcinomas in situ (Bowen type). We sequenced both *TMC6/8* for disease-causing mutations and quantified levels of gene expression. We also performed a systematic literature review to discuss these patients in the context of previously reported cases, mutations already identified, as well as HPV types.

Results: Three patients of one family carried a homozygous splice site mutation in *TMC8* resulting in aberrantly spliced transcripts that were not degraded. By contrast, no *TMC6/8* mutation was detected in the patient from the other family. A systematic literature review revealed 501 described EV patients. Around 40% of EV patients analysed for genetic alterations carried no mutation in *TMC6/8.* While β-HPVs were identified in the majority of cases, α-HPVs were detected in several individuals.

Conclusion: The relatively high proportion of EV patients without mutation in *TMC6/8* indicates the existence of EV-causing mutations in additional, presently unknown gene(s). However, a homozygous *TMC8* splice site mutation in our patients resulted in aberrant transcripts which cannot retain the healthy phenotype. The literature review revealed that HPV-5 is the most commonly identified HPV in EV patients, but HPV-3, HPV-14, and HPV-20 were unexpectedly identified more frequently than HPV-8.

Introduction

Epidermodysplasia verruciformis (EV) (OMIM 226400) is a rare, autosomal recessive genodermatosis with unknown prevalence. EV patients develop plane wart-like lesions during childhood and have a high risk for early onset development of non-melanoma skin cancer (NMSC) on UV light exposed skin. Biallelic loss-of-function (LoF) mutations in either *TMC6* or *TMC8*, also named *EVER1* or *EVER2*, both located on chromosome 17q, are causative for EV[1,2]. Complete loss of TMC8 protein has been shown in two cases[3]. On the other hand, several patients without *TMC6/8* mutation are reported. Lesions of EV patients were shown to harbour high copy numbers per cell of cutaneous human papilloma viruses (HPV), mainly β-HPV, which are assumed to be carcinogenic[4,5]. Understanding the association between different HPV types and malignant transformation better would contribute to development of improved treatments, but very little is known about the frequency of different HPV types in EV patients or about differences in HPV infection between patients and the general population. In this study, which has been approved by the local ethics committee (EKNZ), we describe the clinical and genetic background of four patients with classical phenotype of EV. By reviewing the literature, we furthermore address the open questions regarding the total number of published EV patients, the proportion of families that carry deleterious mutations in *TMC6/8*, and the HPV types identified.

Case 1

A Swiss woman aged 49 was examined. Her parents were first cousins. Since early childhood, reddish skin lesions on the back of her hands and the extensor sides of her knees were visible. The lesions extended to the retroauricular area, neck, chest, arms, hands, knees, and lower legs (Fig. 1a-b). They showed the typical histological appearance of plane warts with minimal hyperkeratosis, acanthosis, as well as some cells with perinuclear halos and bluish staining (Fig. 1c). The patient had few persistent palmar lesions similar to palmar pits which were dermatoscopically consistent with plane warts (Fig. 1d). This is a very rare feature of EV which has been described in only three patients so far[6-8]. The patient developed multiple cutaneous squamous cell carcinomas (cSCC) and carcinomas in situ (Bowen type),

mainly on her forehead and nose (Fig. 1e). After the patient provided informed consent, blood samples and skin biopsies were taken. The whole coding sequence of *TMC6* and *TMC8*, as well as adjacent intronic regions, was sequenced as described elsewere[1]. Some common SNPs without effect on the protein sequence were detected, as well as a heterozygous SNP in *TMC8* (rs7208422, c.917A>T, p.Asn306Ile, MAF = 0.45), but no LoF mutation was found. In a skin biopsy, HPV type 5 was detected by using nested PCR with primers specific for the *L1* capsid gene and subsequent Sanger sequencing.

Cases 2-4

Three Turkish siblings aged 12, 17, and 18 years, as well as their healthy parents, who were second-degree cousins, were included in our study. The siblings developed plane warts during early childhood (Fig. 2a-b). The lesions had an appearance typical for EV and histopathological examination confirmed the diagnosis based on the presence of so-called blue cells (Fig. 2c). These three patients have not yet developed any cancerous lesions, probably because of their young age. Two aunts and one uncle of the patients were affected by EV as well. Blood samples were obtained from the siblings and their parents after they provided informed consent. Sanger sequencing of all coding exons, as well as adjacent intronic regions of *TMC6* and *TMC8*, revealed a homozygous splice site mutation in the donor splice site of IVS9 of *TMC8* (c.1127+1G>C) in all patients (Fig. 2d). Both parents were heterozygous carriers of the identified mutation. Exon-overlapping RT-PCR on mRNA from blood including exon 9 and 10 yielded no product in EV-affected patients, while we obtained the expected PCR-product in parents and all control individuals. Quantitative RT-PCR (qRT-PCR) was performed on *TMC6* exons 4-5 and 10-11, as well as on *TMC8* exons 1-2, 7-8, 9-10, and 14-15. Results were normalized by using *GAPDH*, *TBP*, and *GUSB* with qbase+ (Biogazelle, Belgium). All examined exons of *TMC6*, as well as exons 1-2, 7-8, and 14-15 of *TMC8*, showed stable expression in the patients, their parents and the controls. However, exons 9-10 of *TMC8* could not be amplified in any affected child, and expression was reduced in heterozygous parents compared to controls (Fig. 2e). To identify the main aberrant splice products, RT-PCR products of *TMC8* exons 6-11 were investigated by Sanger sequencing. All

splice products identified in the patients lacked complete exon 9, and a subset additionally lacked exon 10 (Fig. 2f). The former transcript is predicted to result in a frameshift and premature stop codon after 30 amino acids, while the latter transcript would result in an in-frame deletion of 88 amino acids. With the methods described above, infections with β-HPV (type 5 and 9) were detected in all three patients' plane warts.

Discussion

All cases presented in this study showed the typical clinical, histopathological, and viral phenotype of EV. In addition to these characteristic EV features, one individual presented palmar warts, which constitutes a rarely described feature of EV. This patient was affected by diverse NMSCs, including a cSCC on the side of her nose. Since she had never developed plane warts in this region before, a pre-existing lesion does not seem to be a prerequisite for development of NMSC. Reports about the frequency of precanceroses and NMSCs in EV range from none[10] to numerous[11]. Our systematic literature review summarizes cases with congenital EV reported to date, including the novel patients described in this study (table 1). Patients were excluded if they had acquired EV (onset after HIV infection or immunosuppression) or had a very atypical phenotype (excessive wart growth). In total, 501 cases belonging to 347 families have been described with slightly more male patients. The actual number of diagnosed individuals is probably even higher since not every case is presumably published. For example, a clinic in Iraq observed 4-6 affected families per year, mostly from two areas, without reporting details[12], and Japanese clinics reported 66 patients in a questionnaire survey[13]. There are no conclusive studies about the life expectancy of EV patients. However, under regular treatment of precanceroses and NMSCs, patients can reach old age[11].

Despite the EV-typical appearance of patient 1, we were unable to detect any LoF mutation in *TMC6* or *TMC8.* This was in contrast to the second family (cases 2-4), in whom we detected a splice site mutation in *TMC8.* Nevertheless, we were able to measure regular amounts of aberrantly spliced *TMC8* transcripts, which is consistent with a recent report of regular level of

transcripts with in-frame deletions and protein expression in one patient with splice site mutations in *TMC8*[10]. These findings exclude the mechanism of nonsense mediated decay (NMD), which has been reported in patients with *TMC8* premature stop mutation[3]. In contrast to these cases, our patients have not developed any NMSC so far. Moreover, the other EV patient with splice site mutations and stable aberrant transcripts did not develop NMSC either[10]. These findings suggest that the stable transcript and evading NMSC are potentially correlated, which needs to be proven in further studies and a follow-up of our young patients. Since 2002, the *TMC6* and *TMC8* genes have been analysed in 32 families, but in approximately 40% of them (13 families), no LoF mutation could be detected. These findings suggest mutations of at least one more gene connected to EV. Nearly all families with either *TMC6* or *TMC8* deficiency carry a homozygous private mutation indicating consanguinity, which is reported in about half of all patients.

HPV analyses in samples from 208 patients showed a positive result in all except one case[14]. HPV type was determined in 180 of these cases (table S1). Beta-HPV was detected in 159 patients, α-HPV in 57 patients, and γ-HPV in one patient (Fig. 3). Most α-HPVs were detected along with β-HPVs. However, some α-HPV infections were reported without co-infection with β-HPV. These reports should be interpreted with caution. In some studies, the methods used had been developed to find α-HPVs, but were unable to detect β-HPVs. Other studies analysed only perianal samples. In several older studies, HPV-3 was detected by using methods relying on DNA hybridisation or restriction enzyme digestion, without amplification or sequencing. These methods had limited sensitivity and did not differentiate between all types known today. Nevertheless, a recent report has described one individual with a cutaneous HPV-10 (α-HPV) infection without concurrent β-HPV infection, despite a methodical search[16]. HPV-5 and HPV-8 are often mentioned as the most frequent EV-related HPV types. In contrast to that current opinion, our literature review shows that HPV-14 and HPV-20 have been reported more frequently than HPV-8, which shows the need to pay attention to HPV types other than 5 and 8 in future studies. In addition, EV lesions were shown to be coinfected with β-HPVs and polyomaviruses[17]. We conclude that further investigations on the effect of β-

HPV types and polyomaviruses on malignant transformation are necessary to better understand the relation of EV and HPV.

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Conflict of interest

None declared.

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Figures

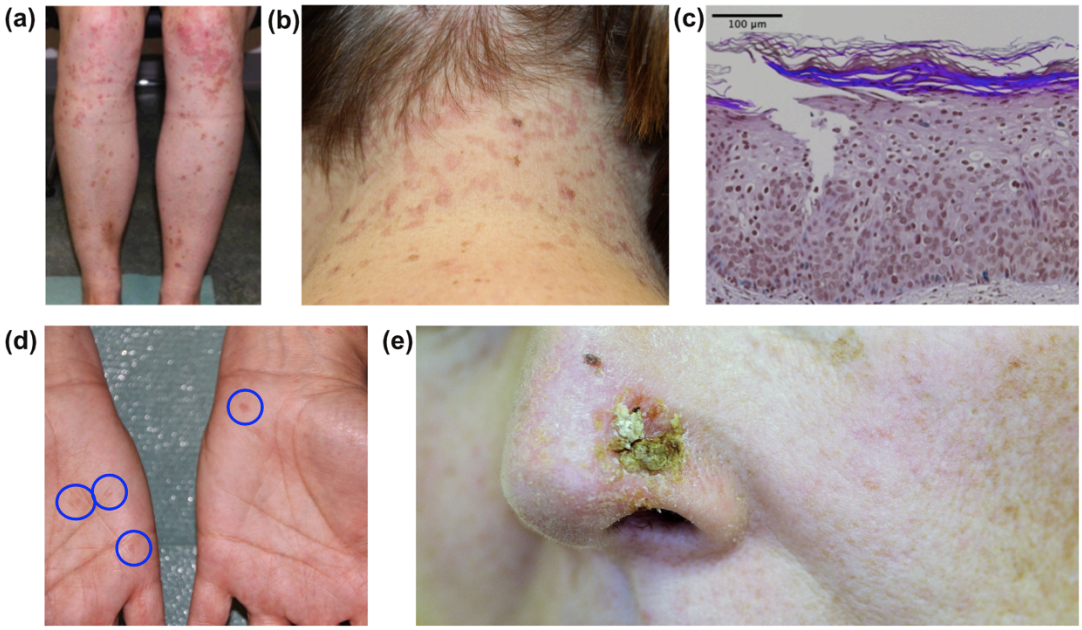

Figure 1: EV lesions on (a) knees and (b) neck of patient 1. (c) Haematoxylin-eosin (HE) stained Bowenoid lesion. Typical pale blue cytoplasm and slightly polymorphic keratinocytes are visible. (d) As a rare feature, the patient showed persistent palmar lesions (marked with blue circles). (e) cSCC developing on the nose of the patient where no previous EV lesions had been present.

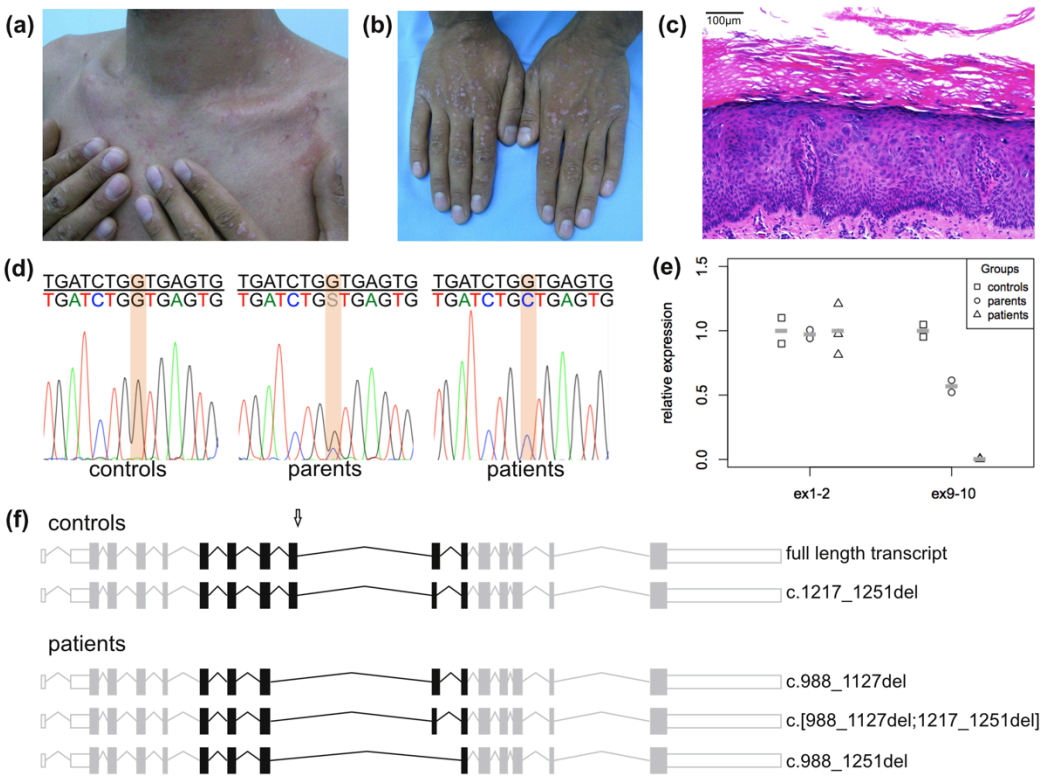

Figure 2: EV lesions on (a) chest and (b) hands of patient 2. (c) HE stained EV lesion of patient 4 with typical blue cytoplasm of keratinocytes and slightly enlarged and hyperchromatic cells mainly in stratum granulosum. (d) Sanger sequencing of *TMC8* revealed a mutation at the donor splice site of intron 9 (*TMC8* c.1127+1G>C), which was found to be homozygous in all three affected siblings and heterozygous in the parents. A wildtype control is shown. (e) Relative expression of exons 1-2 and 9-10 of *TMC8* measured by qRT-PCR. Each symbol represents the average of one individual measured in three replicates. The grey horizontal bars indicate the group average. Whereas no expression could be detected for exon 9-10, a regular expression level is observed for exon 1-2. (f) *TMC8* splice products identified in controls and patients. The region covered by sequencing is indicated in black. The splice site mutation is indicated by an arrow. In healthy controls as well as in patients, a splice variant with a shortened exon 10 can be found additionally to products with full length exon 10, corresponding to ENST00000590184.1[9]. All identified splice products in the patients are lacking at least exon 9, a subset additionally lacks exon 10.

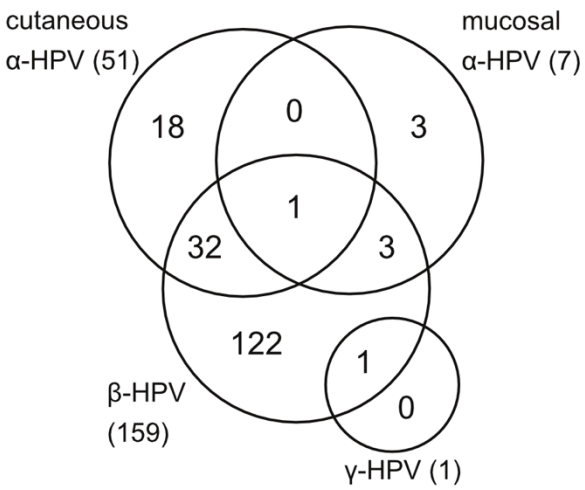

Figure 3: Diagram showing the number of patients by detected HPV infections. Most patients were solely infected by cutaneous β-HPV but some were co-infected by cutaneous or mucosal α-HPV. Only once γ-HPV were identified and in three samples only mucosal α-HPV were detectable.

Tables

Table 1:

Summary of congenital EV cases reported in the literature, including the presented patients. All percentages were calculated respective to all patients or families for whom the information is available.

Number of published cases	501 patients from 347 families
male / female	229 (60.7%) / 148 (39.3%) patients
consanguineous parents	65 families
non consanguineous parents	68 families
HPV detection positive / negative	208 / 1 patients
HPV type determined	180 patients
α-HPV detected	57 (31.7%)
β-HPV detected	159 (88.3%)
γ-HPV detected	1 (0.6%)
TMC6 and *TMC8* sequenced	32 families
LoF mutations in *TMC6*	8 (25.0%)
LoF mutations in *TMC8*	11 (34.4%)
no mutation in *TMC6/8*	13 (40.6%)

Supporting information

Table S1:

Number of EV patients with different HPV-types and species[15] without consideration of combinations in single patients. In total, HPV detection had been performed in 209 patients and many patients were positive for more than one HPV type. The various studies used different methods for HPV detection with different sensitivities and detection ranges.

number of patients	HPV type	species
78	5	β1
41	3	α2
36	20	β1
32	14	β1
31	8	β1
28	undetermined type	-
26	17	β2
19	25	β1
15	24	β1
13	9	β2
11	38	β2
10	19, 23	β1, β2
8	12, 21, 36	β1, β1, β1
7	15, 22	β2, β2
5	47	β1
3	2, 10	α4, α2
2	16, 28, 49, 57, 93	α9, α2, β3, α4, β1
1	4, 6, 11, 18, 27, 32, 34, 37, 53, 75, 76, 92, 96, 111	γ1, α6, α6, α7, α4, α1, α11, β2, α10, β3, β3, β4, β5, β2
1	no HPV detected	-

Number of EV patients described	additional cases in anamnesis reported by patients	Number of families	male	female	consanguineous parents	HPV detected	pathogenic mutation detected	References
1		1	0	1	yes	5	none (TMC examined)	This study.
3		1	2	1	yes	5; 5; 9	TMC8	This study.
3		1				yes; yes; yes		Correa RM, Vladimirsky S, Heideman DA, Coringrato M, Abeldaño A, Olivares L, Del Aguila R, Alonio LV, Snijders PJ, Picconi MA. Cutaneous human papillomavirus genotypes in different kinds of skin lesions in Argentina. *J Med Virol* 2016. doi: 10.1002/jmv.24631.
1		1	0	1	no			Kim C, Hashemi P, Caglia M, Shulman K. Treatment of Imiquimod Resistant Epidermodysplasia Verruciformis With Ingenol Mebutate. *J Drugs Dermatol* 2016. **15:**350-2.
1		1	1	0		8, 22, 47	TMC8	Miyauchi T, Nomura T, Suzuki S, Takeda M, Shinkuma S, Arita K, Fujita Y, Shimizu H. Genetic analysis of a novel splice-site mutation in TMC8 reveals the in-vivo importance of the TMC domain of TMC8. *Br J Dermatol* 2016. **DOI:**10.1111/bjd.14569
1		1	1	0				Arora T, Sharma S, Sharma N, Titiyal JS. Bilateral recurrent ocular surface squamous cell cancer associated with epidermodysplasia verruciformis. *BMJ Case Rep* 2015. doi: 10.1136/bcr-2014- 207495
1		1	0	1		20	none (TMC examined)	Kehdy J, Erickson C, Rady P, Tyring S, Gaspari AA. Epidermodysplasia verruciformis: successful treatment with squaric acid dibutylester. *Cutis* 2015. **96:** 114-8
1		1	1	0		16	TMC8	Mizuno Y, Kato G, Shu E, Ohnishi H, Fukao T, Ohara O, Fukumoto H, Katano H, Seishima M. Merkel cell polyomavirus-positive Merkel cell carcinoma in a patient with epidermodysplasia verruciformis. *Acta Derm Venereol* 2015. **95:** 98–9
1		1	0	1	yes		none (TMC examined)	O'Blenes C, Pasternak S, Issekutz A, Gillis J, Chowdhury D, Finlayson L. Epidermodysplasia verruciformis in lipoid proteinosis: case report and discussion of pathophysiology. *Pediatr Dermatol* 2015. **32:** 118-21
1		1	1	0	no	20, 23, 27	none (TMC examined)	Borgogna C, Landini MM, Lanfredini S, Doorbar J, Bouwes Bavinck JN, Quint KD, de Koning MNC, Genders RE, Gariglio M. Characterization of skin lesions induced by skin-tropic α- and β-papillomaviruses in a patient with epidermodysplasia verruciformis. *Br J Dermatol* 2014. **171:** 1550–4
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1		1	1	0				Rajabi MT, Ghasemi H, Safizadeh M, Jamshidi S, Asadi-Amoli F, Abrishami Y, Oestreicher JH. Conjunctival squamous cell carcinoma with intraocular invasion after radiotherapy in epidermodysplasia verruciformis. *Can J Ophthalmol* 2014. **49:** e43–6
1		1	1	0				Sharma S, Barman KD, Sarkar R, Manjhi M, Garg VK. Efficacy of oral zinc therapy in epidermodysplasia verruciformis with squamous cell carcinoma. *Indian Dermatol Online J* 2014. **5:** 55-8
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1		1	1	0	no			Kumar P, Lal NR, Gharami RC. Palmar pits and epidermodysplasia verruciformis: a rare association. *Indian J Dermatol Venereol Leprol* 2012. **78:** 501-3
1		1	1	0		10		Zahir A, Craig L, Rady P, Tyring S, Ehrlich A. Epidermodysplasia verruciformis associated with HPV 10. *Dermatol Online J* 2013. **19:** 2
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1		1	1	0		3, 14, 18	TMC6	Sunohara M, Ozawa T, Morimoto K, Harada T, Ishii M, Fukai K. Dye laser photodynamic therapy for Bowen's disease in a patient with epidermodysplasia verruciformis. *Osaka City Med J* 2012. **58:** 77-8
2		1	2	0	yes			Bhutoria B, Shome K, Ghosh S, Bose K, Datta C, Bhattacharya S. Lewandowsky and Lutz dysplasia: report of two cases in a family. *Indian J Dermatol* 2011. **56:** 190-3
1		1	0	1	yes	16		Hayashi S, Hatamochi A, Soutome A, Hamasaki Y, Yamazaki S, Kawase M, Tadano M, Kitasato H. A case of epidermodysplasia verruciformis (EV) with human papillomavirus 16 (HPV16) DNA detected in the skin lesions: can HPV16 infect patients with EV?. *Int J Dermatol* 2011. **50**: 1168-70
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1		1	1	0		5, 8, 9, 12, 14, 17, 19, 21, 23, 24, 25, 36, 37, 38, 47, 49, 75, 76	none (TMC examined)	Dell'Oste V, Azzimonti B, De Andrea M, Mondini M, Zavattaro E, Leigheb G, Weissenborn SJ, Pfister H, Michael KM, Waterboer T, Pawlita M, Amantea A, Landolfo S, Gariglio M. High beta-HPV DNA loads and strong seroreactivity are present in epidermodysplasia verruciformis. *J Invest Dermatol* 2009. **129:** 1026-34
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