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Cutaneous epithelioid haemangiomas show somatic mutations in the mitogen-activated protein kinase pathway

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Summary

Background Epithelioid haemangioma (EH) arising from the skin is a benign vascular tumour with marked inflammatory cell infiltration, which exhibits a high tendency to persist and frequently recurs after resection. So far, the underlying pathogenesis is largely elusive.
Objectives To identify genetic alterations by next-generation sequencing and/or droplet digital polymerase chain reaction (ddPCR) in cutaneous EH.
Methods DNA and RNA from an EH lesion of an index patient were subjected to whole-genome and RNA sequencing. Multiplex PCR-based panel sequencing of genomic DNA isolated from archival formalin-fixed paraffin-embedded tissue of 18 patients with cutaneous EH was performed. ddPCR was used to confirm mutations.
Results We identified somatic mutations in genes of the mitogen-activated protein kinase (MAPK) pathway (*MAP2K1* and *KRAS*) in cutaneous EH biopsies. By ddPCR we could confirm the recurrent presence of activating, low-frequency mutations affecting *MAP2K1*. In total, nine out of 18 patients analysed showed activating MAPK pathway mutations, which were mutually exclusive. Comparative analysis of tissue areas enriched for lymphatic infiltrate or aberrant endothelial cells, respectively, revealed an association of these mutations with the presence of endothelial cells.
Conclusions Taken together, our data suggest that EH shows somatic mutations in genes of the MAPK pathway which might contribute to the formation of this benign tumour.

What is already known about this topic?

- Epithelioid haemangioma (EH) arising from the skin is a benign vascular tumour of unknown aetiology.

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Conflicts of interest
V.G. has received honoraria from Bristol-Myers Squibb (BMS) and reports travel support from BMS, Merck Sharp & Dohme (MSD), Novartis, Pierre Fabre Pharmaceuticals and Sanofi Genzyme; outside the submitted work. S.F. reports consulting or advisory board membership for Bayer, Illumina and Roche; honoraria from Amgen, Eli Lilly, PharmaMar and Roche; research funding from

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K.M. and C.K. contributed equally to this work.

Data availability statement

Data will be provided upon request for reasonable academic studies by the corresponding author.

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- Cutaneous EH often shows a marked inflammatory infiltrate indicating a reactive origin.

What does this study add?

- Half of the samples from cutaneous EH in this study showed activating mutations in the mitogen-activated protein kinase pathway (*MAP2K1* and *KRAS*).
- Mutations were mutually exclusive.

What is the translational message?

- Somatic mutations seem to contribute to the formation of a significant proportion of cutaneous EH.
- The molecular alterations found might be sensitive for targeted therapies.

Epithelioid haemangioma (EH) is a rare benign or low-grade vascular neoplasm. EH mainly affects adults aged 20–50 years of both sexes.[1–3] Apart from the skin, EH may develop in deep soft tissue and bone.[4,5] EH shows a high rate of local recurrence and management of symptomatic EH remains a clinical challenge.

Histologically, EH consists of well-formed vessels lined by prominent epithelioid endothelial cells.[3] At present, three major histological EH variants are recognized descriptively: conventional (typical) EH, cellular EH and angiolymphoid hyperplasia with eosinophilia (ALHE). Cutaneous EH presents as small, often multiple, red-to-purple papules, plaques or nodes which tend to be asymptomatic but occasionally may be painful or itching.[6] When multiple, EHs usually appear grouped and may be confluent. Some clinical and histological findings indicate a reactive nature of EH,[1,7] such as history and evidence of previous inflammatory stimuli or trauma. However, the aetiology and pathogenesis of EH are still unclear.

For EH found in connective tissue, Antonescu et al.[4] described gene rearrangements affecting *FOS* and *FOSB*[3,4,8] and consecutive overexpression of their transcripts. As immediate early gene products, FOS proteins have been implicated as regulators of cell proliferation, differentiation, transformation, tumour invasion, distant metastasis and angiogenesis.[9,10] While rearrangements of *FOS* family members are frequently associated with osseous EH, they are rarely found in cutaneous EH.[4,8] Nevertheless, a strong nuclear expression of FOSB protein is commonly found in the epithelioid endothelia of cutaneous EH biopsies.[2,11,12] Of note, the mitogen-activated protein kinase (MAPK) pathway plays an important role in the expression, stability and transactivating function of immediate early gene products, which includes the FOS family members.[10] Based on these observations and whole-genome sequencing (WGS) of an index patient with recurrent head and neck EH (ALHE subtype), we hypothesized that cutaneous EH shows activating mutations in the MAPK pathway.

Materials/patients and methods

Patients and samples

The index patient was enrolled into the DKTK MASTER[13] study after written informed consent for molecular analysis approved by the Ethics Committee of the Medical Faculty of Heidelberg University (S-206/2011). For additional patients, informed consent was waived by the Ethics Committee of the University Hospital Würzburg due to the retrospective nature of the study and the collection of anonymous patient data generated during routine care. No healthy control tissue was available. Clinical data (age, sex, localization) were obtained by chart review (Table 1). For publication of patient photos, written informed consent was obtained from the index patient.

Histopathology and immunohistochemistry

The study included 18 patients with cutaneous EH diagnosed according to the current World Health Organization recommendations.[14] Histological/morphological diagnosis of EH was established by two independent pathologists (H.Ku., H.Kn.) and confirmed by a third (A.R.). Histological subclassification (conventional EH, cellular EH, ALHE) was conducted based on the criteria defined previously[8,15] (Table S1; see Supporting Information). Immunohistochemical stainings of phosphorylated ERK1/2 ($P^{Thr202/Tyr204}$-p44/42 MAPK, D13·14·4E, 1 : 800, Cell Signaling, Leiden, the Netherlands; Figure S1; see Supporting Information), ERG (EP111, 1 : 2000, Medac, Wedel, Germany) and $KRAS^{Q61R}$ [Anti-NRAS (mutated Q61R) antibody, SP174, 1 : 20, Abcam, Cambridge, UK] were performed using the HRP HiDef 2-Step Polymer Detection System (Medac) according to the manufacturer's recommendation. For the $NRAS^{Q61R}$ antibody, cross-reactivity was previously described.[16,17] In this study, a $KRAS^{Q61R}$-positive colorectal carcinoma was used as a positive control, whereas EH-01B

Table 1 Patient characteristics and detection of MAPK pathway-associated mutations by multiplex-polymerase chain reaction (PCR)-based panel sequencing and droplet digital PCR (ddPCR)

Sample ID	Clinical site	Sex	Age	MAPK signalling-associated mutation	Detected by NGS (allele frequency, %)	Detected by ddPCR (allele frequency, %)
EH-01A[a]	Scalp	F	51	*MAP2K1*: c.383G>A, p.Gly128Asp[c]	+ (1·5%)	+ (1·3%)
EH-01B[a]	Scalp	F	51	–	– (0·3%)[g]	– (0·1%)[g]
EH-02	Pre-auricular	F	78	*MAP2K1*: c.171G>T, p.Lys57Asn[d]	+ (1·9%)	+ (1·0%)
EH-03	Eyebrow	F	42	*MAP2K1*: c.171G>C, p.Lys57Asn[e]	+ (2·0%)	+ (1·0%)
EH-04	Retro-auricular	M	43	–	–	–
EH-05	Scalp	M	21	–	–	–
EH-06	Upper arm	M	47	*MAP2K1*: c.171G>C, p.Lys57Asn[e]	+ (6·2%)	+ (5·2%)
EH-07	Nose	F	38	*MAP2K1*: c.171G>T, p.Lys57Asn[d]	–	+ (0·4%)
EH-08	Scalp	F	26	–	–	–
EH-09	Forehead	M	36	–	–	–
EH-10	Upper arm	M	25	–	–	–
EH-11	Left shoulder	M	49	–	–	–
EH-12	Scalp	M	36	–	–	–[h]
EH-13[a]	Neck	F	51	*MAP2K1*: c.383G>A, p.Gly128Asp[c]	+ (2·2%)	+ (1·7%)
EH-14	Left eye/nose	F	40	*MAP2K1*: c.383G>A, p.Gly128Asp[c]	– (0·3%)[g]	+ (0·3%)
EH-15	Face	F	45	*MAP2K1*: c.171G>C, p.Lys57Asn[e]	–	+ (1·1%)
EH-16	Penis	M	39	–	–	–
EH-17	Forehead	M	67	*MAP2K1*: c.171G>T, p.Lys57Asn[d]	–	+ (0·8%)
EH-18[b]	Upper lip	F	64	–	–	–
EH-19[b]	Right cheek	F	64	–	–	–
EH-20	Forearm	M	86	*KRAS*: c.182A>G, p.Gln61Arg[f]	+ (6·0%)	Not performed

Mutated allele frequencies of the respective missense variants are indicated. [a]Samples EH-01A+B and EH-13 represent recurring EH lesions of the same patient obtained at different timepoints; [b]Samples EH-18 and EH-19 are solitary lesions of a grouped EH from the same patient; [c]NM_002755·3 (exon3); [d]NM_002755·3 (exon2); [e]NM_002755·3 (exon2); [f]NM_004985·3 (exon3); [g]The mutation was detected below the cutoff [with an alternative read depth of 10 × (EH-14) or lower limit of the 95% confidence interval > 0·2% (EH-01B)]; [h]Sample EH-12 was excluded from ddPCR analysis of *MAP2K1* c.383G>A mutation due to high frequency of C>T transitions visible in NGS analysis. EH, epithelioid haemangioma; MAPK, mitogen-activated protein kinase; NGS, next-generation sequencing; +, mutant alleles were detected; –, no mutant alleles were detected.

and EH-05 served as negative control samples (Figure S2; see Supporting Information).

Whole-genome sequencing and RNA sequencing

Processing of cryopreserved tumour and control specimens of the index patient[18] as well as WGS, mapping of sequencing data and detection of variants was performed as described.[19] Additionally, RNA sequencing libraries were prepared using the TruSeq stranded RNA Sample Preparation Kit v2 (Illumina, San Diego, CA, USA). RNA was sequenced on the Illumina HiSeq4000. RNA sequencing reads were mapped with STAR (21) version 2.5. The variant in *MAP2K1* was detected in both DNA and RNA by an additional whitelisting step that looks up genomic positions for which pathogenic or likely pathogenic point mutations are described in OncoKB.[20] For this, genomic coordinates were inferred from amino acid positions extracted from OncoKB using Gencode (Human release 19) and dbNSFP 2·9.[21] Tabix was used to screen these positions in the raw vcf file created by samtools mpileup and bcftools view with options -vcgN -p 2·0 to report all positions with at least one nonreference read. Variants supported by fewer than two reads were discarded. Detected variants were manually checked and displayed using the integrative genomics viewer.[22]

DNA extraction

Formalin-fixed paraffin-embedded (FFPE) tissue specimens of patients with cutaneous EH were microdissected to enhance the number of atypic endothelial cells. Genomic DNA was extracted with the GeneRead DNA FFPE Kit according to the manufacturer's recommendation (Qiagen, Hilden, Germany) and the Maxwell RSC Blood DNA Kit after a pretreatment with a THG1-Thioglycerol/incubation buffer mix for 10 min at 80°C and subsequent incubation with proteinase K at 65°C overnight (Promega, Walldorf, Germany).

Multiplex-polymerase chain reaction-based panel sequencing

Next-generation sequencing

Concentration of amplifiable genomic DNA was determined by quantitative polymerase chain reaction (qPCR) (TaqMan RNase P Detection Reagents Kit, ThermoFisher, Waltham, MA, USA).

Twenty-one DNAs of 18 patients met quality criteria and were processed further. Libraries were prepared by a multiplex PCR approach using the Oncomine Focus Assay and the Ion AmpliSeq Library Kit 2·0 (both ThermoFisher, Table S2; see Supporting Information). Libraries were templated and enriched with the Ion OneTouch 2 and the Ion OneTouch ES automated systems (both ThermoFisher). Sequencing was performed using semiconductor sequencing technology (Ion GeneStudio S5, ThermoFisher) with a mean sequencing depth of 2262x (Table S3; see Supporting Information).

Somatic variant calling

Bam-files were generated by Torrent Suite software, version 5.10 (ThermoFisher). Readgroups were added to the bam-files using Picard, version 1.125 (available online at: http://broadinstitute.github.io/picard/) and SAMtools, version 1.38,[23] was used for index creation. Coverage calculations were performed with GATK, version 3.59.[24] MuTect2,[25] version 4.0.11.0, which is integrated in the GATK package, was used for somatic variant calling with the disable-tool-default-read-filters set to true. All variants were annotated with ANNOVAR, version 2017-06-19.[26] Variant filtering was adapted depending on the limited tumour cell amounts. The minimum frequency of the altered allele was set to 1% with at least 15-fold coverage. Moreover, we filtered for clinically relevant hotspot mutations, which were characterized as likely pathogenic or pathogenic in the ClinVar database (NCBI, Bethesda, MD, USA). All detected mutations were manually inspected in the integrative genomics viewer.

Droplet digital polymerase chain reaction

Extracted DNA from 21 cutaneous EH tissue samples was analysed by droplet digital PCR (ddPCR) using the assay *MAP2K1* p.G128D, Human (c.383G>A; dHsaMDS123709391) for p.Gly128Asp-mutated and custom assays for p.Lys57Asn-mutated MEK1 (c.171G>T and c.171G>C; sequences in Table S4; see Supporting Information) according to the manufacturer's instructions (Bio-Rad, Feldkirchen, Germany). Fluorescent measurement of the droplets was conducted with the QX200 Droplet Reader and analysed with QX Manager Software (Bio-Rad). gBlocks® DNA fragments with the mutation of interest (Integrated DNA Technologies, Coralville, IA, USA) were used as positive controls for the custom-made *MAP2K1* c.171G>T/C ddPCR assays and gating was performed based on positive and negative controls. Detection of a *MAP2K1* mutation was always validated in at least two independent experiments and the mutant allele frequency along with the corresponding Poisson-based 95% confidence intervals (CI) were calculated based on the merged datasets. Cases with a lower limit of the 95% CI exceeding 0·2% fractional abundance were considered mutation-positive. EH-12 was manually excluded from the assessment of a *MAP2K1* c.383G>A mutation due to a high incidence of C>T transitions in the next-generation sequencing (NGS) analysis suggesting poor quality and FFPE artefacts of the DNA sample.

Cell culture, *MAP2K1* mutagenesis and lentiviral gene transfer

Primary human umbilical vein endothelial cells (HUVECs; PromoCell, Heidelberg, Germany) were cultured and used until passage 5 as described previously.[27]

The lentiviral expression plasmid for human wildtype *MAP2K1* (NM_002755·4; pCDH puro MEK1 WT) was kindly provided by PD Dr Roland Houben (University Hospital Würzburg, Germany). The corresponding vectors encoding *MAP2K1 c.171G>T* and *MAP2K1 c.383G>A* were generated by site-directed mutagenesis with the QuikChange Lightning Kit (Agilent, Santa Clara, CA, USA) according to the manufacturer's instructions using the following mutagenesis primers: c.171G>T forward: 5′-cccacct tctgattctgggtaagaaaggcctc-3′, reverse: 5′-gaggcctttcttacccagaatca gaaggtggg-3′; c.383G>A forward: 5′-gaacgcaccatagaagtccacgatg tacggaga-3′, reverse 5′-tctccgtacatcgtggacttctatggtgcgttc-3′. Successful mutagenesis of wildtype MEK1 was confirmed by Sanger sequencing of the lentiviral expression vectors. Lentiviral particles were produced by HEK293T cells as described previously[28] and virus-containing supernatants added to the HUVECs for 6 h. Cells were selected with 2 μg mL^{-1} puromycin (InvivoGen; San Diego, CA, USA). Cells were then cultured in Macrovascular Endothelial Growth medium [1 part EGM™ Endothelial Cell Growth medium (Lonza, Cologne, Germany); 2 parts M199 medium (GlutaMAX, ThermoFisher) supplemented with 10% fetal calf serum, 30 μg mL^{-1} gentamicin, 15 ng mL^{-1} amphotericin B (all Sigma, Taufkirchen, Germany), 200 IU L^{-1} Heparin-Natrium 5000 (Ratiopharm, Ulm, Germany)].

Western blot analysis

Cell lysis and Western blot analysis was performed as described previously[29] using the following primary antibodies: anti-$P^{Thr202/Tyr204}$-ERK1/2 (D13·14·4E, #4370), anti-ERK1/2 (#9102), anti-MEK1 (61B12, #2352), anti-GAPDH (D16H11, #5174) (all Cell Signaling) and anti-Vinculin (V9131; Sigma). For comparison of constructs, cells were harvested 6 days after lentiviral transduction. In experiments involving trametinib, a MEK1/2 inhibitor (Cayman Chemical, Ann Arbor, MI, USA), cells were harvested after 20 days. Trametinib was added at the indicated concentrations for the last 24 h.

Analysis of cellular morphology

The 'Analyze Particles' function of ImageJ was used in three randomly chosen microphotographs per condition to assess cell size. Trametinib had been added for 24 h where indicated. Quantification statistics can be found in Tables S5 and S6 (see Supporting Information). Significance was determined by one-way ANOVA and subsequent Dunnett's multiple comparison test (empty vector as control) or two-way ANOVA with Šidák correction (trametinib treatment).

Results

Oncogenic *MAP2K1* mutation in a patient with recurrent epithelioid haemangiomas/angiolymphoid hyperplasia with eosinophilia (index patient)

In 2013, a 46-year-old female presented herself with a history of multiple local treatments for EH subcategorized as ALHE at her scalp (Figure 1a). From 2013 until the beginning of 2015, she received local and systemic medical treatments. In March 2015, she underwent complete resection of the affected skin/soft tissue. After an interval of approximately 3 years, she developed another local recurrence. At this timepoint, in March 2018, she was enrolled into the MASTER (Molecularly Aided Stratification for Tumor Eradication Research) programme of the German Cancer Consortium[13] and cryopreserved material was subjected to WGS and RNA sequencing. While RNAseq did not indicate gene fusions affecting *FOS(B)*

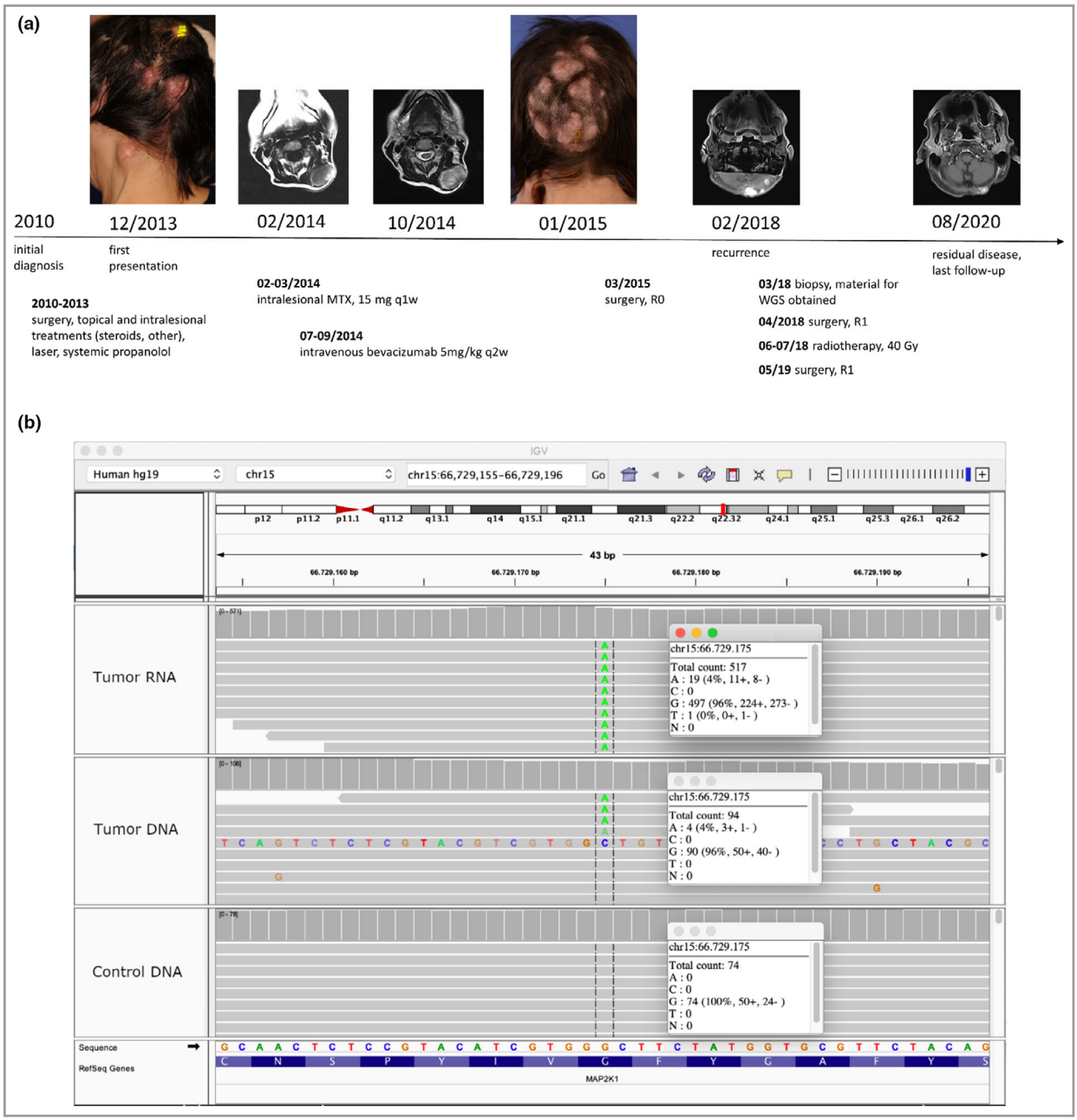

Figure 1 Activating *MAP2K1* mutation in a case of recurrent epithelioid haemangioma (EH). (a) Timeline to illustrate the clinical course of EH in the index patient and timepoints of biopsy collection (EH-01, EH-13). (b) Integrative genomics viewer screenshot showing the alignments of the tumour RNA (upper panel), the tumour DNA (middle panel) and the control DNA (lower panel). The alignments have been sorted by base at the position of the mutation.

or *GATA6-FOXO1*, WGS revealed an oncogenic hotspot mutation in exon 3 of the *MAP2K1* gene (c.383G>A) (Figure 1b), which was detected in the tumour material, but not in healthy control tissue. This mutation was found to be expressed in RNA sequencing data of the patient (Figure 1b) and leads to an amino acid substitution in the MEK1 protein at position 128 (p.Gly128Asp). We then performed targeted panel sequencing of two microdissected regions of archival FFPE material of the same specimen. One area was enriched with blood vessels surrounded by neoplastic plump endothelial cells (EH-01A), while the second region showed a dense lymphoid infiltrate (EH-01B) (Figure 2). Filter settings for variant calling were adapted to the low number of neoplastic cells in the analysed tissue samples. The *MAP2K1* c.383G>A mutation (MEK1 p.Gly128Asp) was detected in the epithelioid cell-enriched specimen with a mutant allele frequency of 1·3%, but not in the lymphocyte-enriched specimen (Tables 1, S3 and S7; see Supporting Information). In line with the strong P-ERK1/2 immunohistochemical reactivity of the aberrant endothelia, a surrogate marker for MAPK pathway activity (Figure 2), this spatial restriction strongly suggests the neoplastic epithelioid endothelial cells as mutation carriers.

Recurrent activating mitogen-activated protein kinase pathway mutations in cutaneous epithelioid haemangiomas detected by targeted next-generation sequencing

To elucidate the prevalence of MEK1 mutations in cutaneous EH, the mutational status of the *MAP2K1* gene was investigated in 19 additional FFPE specimens, which included material from 17 further patients with cutaneous EH as well as a second biopsy from the index patient that had been obtained at an earlier timepoint (Figure 3). Endothelial cells of all samples showed P-ERK1/2 expression indicating MAPK pathway activation (Figure S1). Genomic DNA was extracted and analysed by targeted NGS to detect (likely) pathogenic (driver) mutations within the *MAP2K1* hotspot regions. With this approach, we could confirm the presence of the low-frequency *MAP2K1* c.383G>A mutation in a prior ALHE lesion of the index patient (EH-13 from 2015), demonstrating the persistence of this mutation during disease recurrence (Tables 1 and S7).

In addition to the index patient, specimens obtained from three other patients harboured activating mutations in the investigated *MAP2K1* gene (Tables 1 and S7). These samples showed activating mutations in exon 2 of the *MAP2K1* gene (EH-02, c.171G>T; EH-03 and EH-06, c.171G>C). Both missense variants lead to an identical amino acid exchange at position 57 in the negative regulatory domain (Helix A) of the MEK1 protein (p.Lys57Asn) (Figure 4a). Although Gly128 is located in the core kinase domain of MEK1, three-dimensional *in silico* modelling of the kinase revealed a common *MAP2K1* mutation hot spot in cutaneous EH predicted to affect the inhibitory function of Helix A, leading to a constitutive kinase activity (Figure 4b). Accordingly, overexpression of $MEK1^{Lys57Asn}$ or $MEK1^{Gly128Asp}$ strongly induced MAPK pathway activity in HUVECs as opposed to the wildtype protein (Figure 5a). This was accompanied by an altered morphology of HUVECs reflected by a significant increase in cell size (Table S5, Figures 5b and S3a, b; see Supporting Information) and could be reversed by treatment with a MEK1/2 inhibitor (Table S6, Figures 5c,d and S3c). An additional patient was found to have an oncogenic *KRAS* mutation in exon 3 by targeted NGS (EH-20: c.182A>G, KRAS p.Gln61Arg) (Table 1, S3 and S7). The presence of mutated KRAS protein in endothelial cells could be confirmed by immunohistochemical staining of the corresponding FFPE specimen (Figure 5e, Figure S2). These data indicate that recurrent mutations within the MAPK signalling pathway

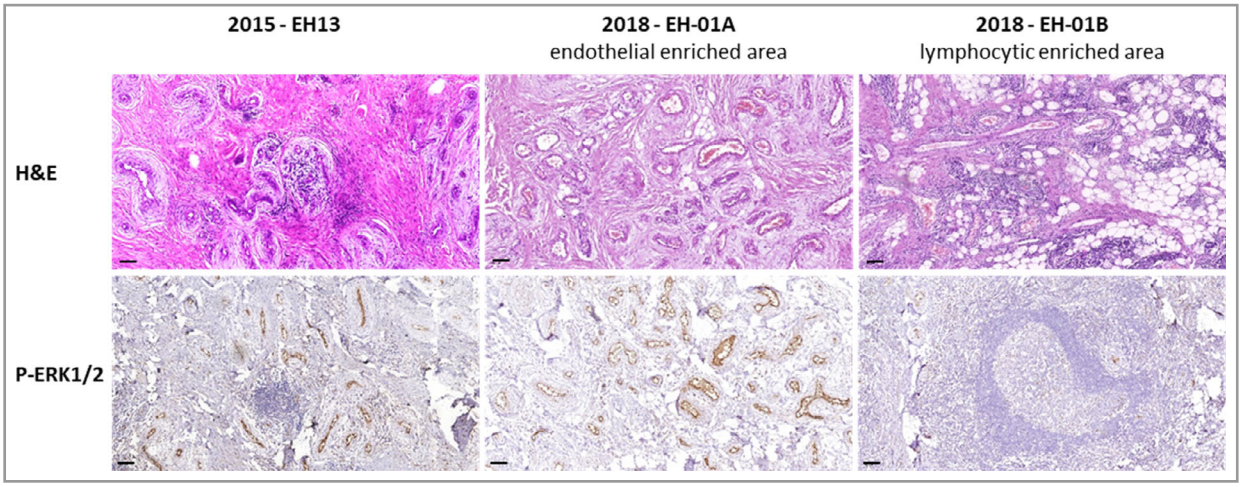

Figure 2 Increased mitogen-activated protein kinase (MAPK) pathway activity in aberrant endothelial cells. Haematoxylin and eosin-based (H&E) histology and immunohistochemical $P^{Thr202/Tyr204}$-ERK1/2 staining of affected tissue specimen (EH-01A, endothelial enriched area; EH-01B, predominant lymphocytic infiltrate; EH-13, prior lesion). Scale bars ≙ 100 μm.

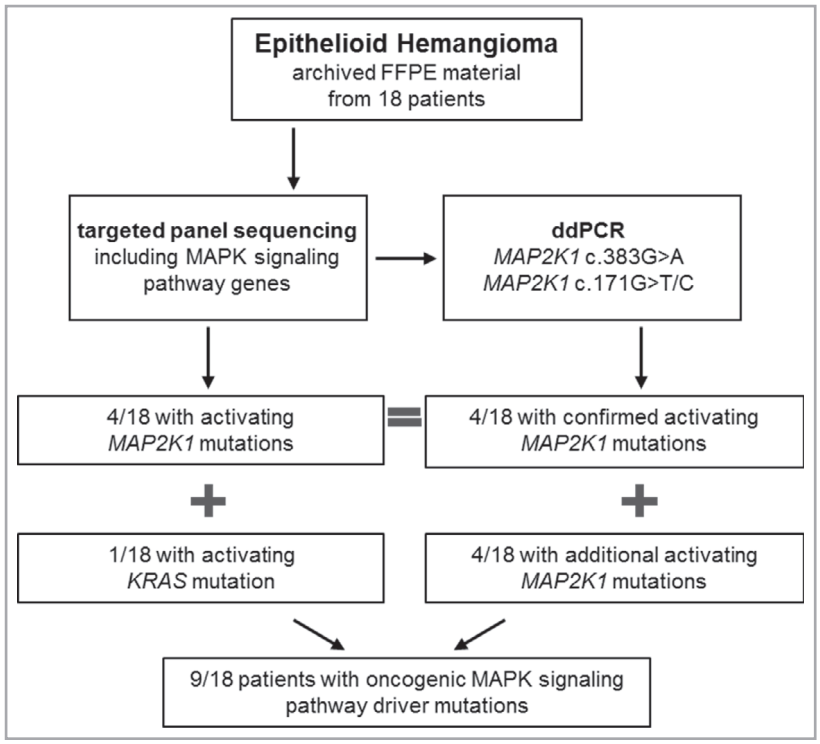

Figure 3 Flowchart of study design and genetic analyses. Targeted panel sequencing showed activating *MAP2K1* and *KRAS* mutations in the genomic DNA from archival formalin-fixed paraffin-embedded (FFPE) epithelioid haemangioma (EH) tissue. Droplet digital polymerase chain reaction (ddPCR) analysis was used to confirm the *MAP2K1* variants in these patient samples and to additionally identify low-frequency *MAP2K1* mutations in further EH biopsies.

might play a role during the pathogenesis of this vascular neoplasm.

Confirmatory and explorative droplet digital polymerase chain reaction analyses of low-frequency activating *MAP2K1* pathway mutations in cutaneous epithelioid haemangiomas

To confirm the recurrently occurring *MAP2K1* mutations with an independent method, we performed ddPCR-based rare allele detection of the identified *MAP2K1* hotspot mutations (c.383G>A, c.171G>T, c.171G>C) in the extracted genomic DNA from all 21 EH tissue samples (Table S8; see Supporting Information). ddPCR analysis not only corroborated the presence of the *MAP2K1* variants detected by NGS, but also revealed low-frequency *MAP2K1* mutations in four additional patients (EH-14: c.383G>A; EH-07, EH-17: c.171G>T; EH-15: c.171G>C) (Table 1). Consequently, activating MAPK pathway mutations could be found in the affected tissue from nine of the 18 patients with EH analysed, independent of their histological subcategory (Table S1). Although the mutant allele frequency did not correlate with the number of atypical endothelial cells in general (data not shown), there was again a clear association with the presence of atypical endothelial cells (EH-01A: 1·3% allele frequency; EH-01B: 0·1% allele frequency; Figure 6) in the lesion of the index patient. Moreover, the presence of the distinct MAPK pathway mutations was mutually exclusive and different lesions (spatial and temporal) of a given patient showed the same status (EH-01, EH-13: c.383G>A; EH-18, EH-19: no mutation detected) (Table 1). This indicates the high specificity of the employed analyses as well as a potential functional role of the detected genomic alterations in the pathogenesis of cutaneous EH.

Discussion

In this study, we were able to discover low-frequency activating mutations in genes of the MAPK pathway in samples from patients with cutaneous EH. Mutations were recurrent, mutually exclusive and temporally conserved.

Detection of low-frequency genetic variants resulting, e.g. from a low frequency of atypical cells, is still challenging. In general, NGS approaches could determine valid mutation profiles of FFPE material down to an allele frequency of approximately 2–3%.[30,31] In our study, all *MAP2K1* mutations detected by NGS were confirmed by ddPCR and oncogenic *MAP2K1* mutations were found in four additional cases by ddPCR. However, we cannot conclude a causative relationship of the low-frequency MAPK pathway mutations and EH. Additional alterations such as cell-specific losses of wildtype alleles might contribute to the pathogenesis.[24] An *in vivo* model and further comprehensive studies addressing fusions previously

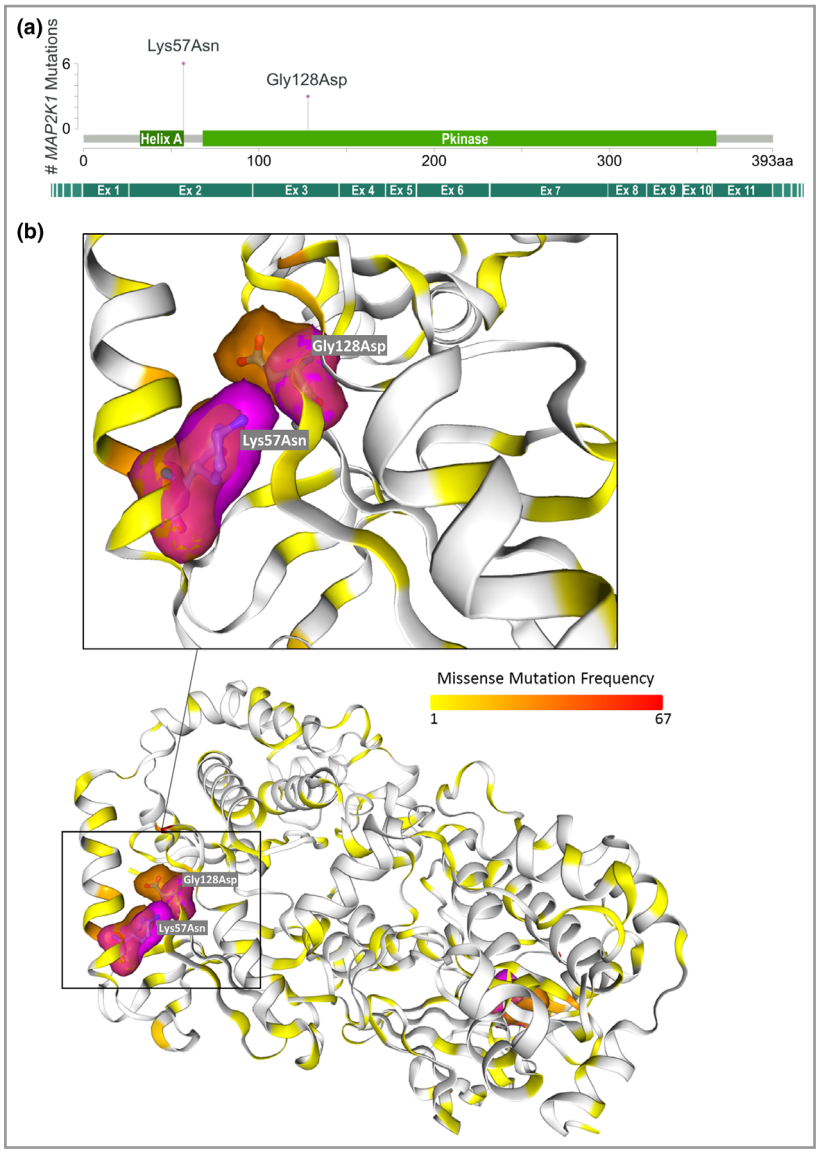

Figure 4 Oncogenic *MAP2K1* variants in epithelioid haemangioma. (a) Mapping of the detected mutations to MEK1 protein domains and schematic *MAP2K1* exon structure (NM_002755). Pkinase, core protein kinase domain; aa, amino acids. (b) Three-dimensional localization of the affected amino acids in the identified oncogenic MEK1 variants visualized by COSMIC-3D (UniProtKB: Q02750). The amino acid alterations (p.Gly128Asp: COSM1374186, p.Lys57Asn: COSM5520914) are depicted as ball-and-stick and surface models in a MEK1 homo-dimer (PDB: 3W8Q). Colour coding indicates missense mutation frequency within MEK1 as reported by the Catalogue Of Somatic Mutations In Cancer (COSMIC).

reported and other genes of the MAPK pathway are needed to understand the genetic landscape and pathogenesis of EH.

The presence of activating mutations in genes of the MAPK pathway is not only a common finding in cancer,[32] but also appears to play an important role in the pathogenesis of various (benign) vascular anomalies ('issva.org/classification'; accessed 20 November 2020). For instance, extracranial arteriovenous malformations are mostly caused by somatic mutations in *MAP2K1* or other genes of the MAPK pathway.[33,34] Pyogenic granuloma, a benign tumour often developing after trauma, also shows activating mutations in the MAPK pathway, including the *BRAF*, *RAS* and *GNA11* genes.[35,36] Our study now adds cutaneous EH to the group of MAPK-associated vascular entities illustrating the central role of this pathway in human diseases.

Two major hypotheses have been proposed on the aetiology of EH: a reactive, inflammatory pathogenesis and a neoplastic origin. For bone and soft tissue EH, *FOS(B)* gene fusions have been identified in a substantial proportion of cases.[4,8] More recently, Antonescu et al. reported *GATA6-FOXO1* fusions in EH lacking *FOS(B)* gene fusions.[37] Based on this and our findings, EH seems to be a vascular neoplasm, showing a marked (secondary) inflammatory infiltrate rather than a reactive lesion, such as Kimura disease.[6] However, its clinical presentation as

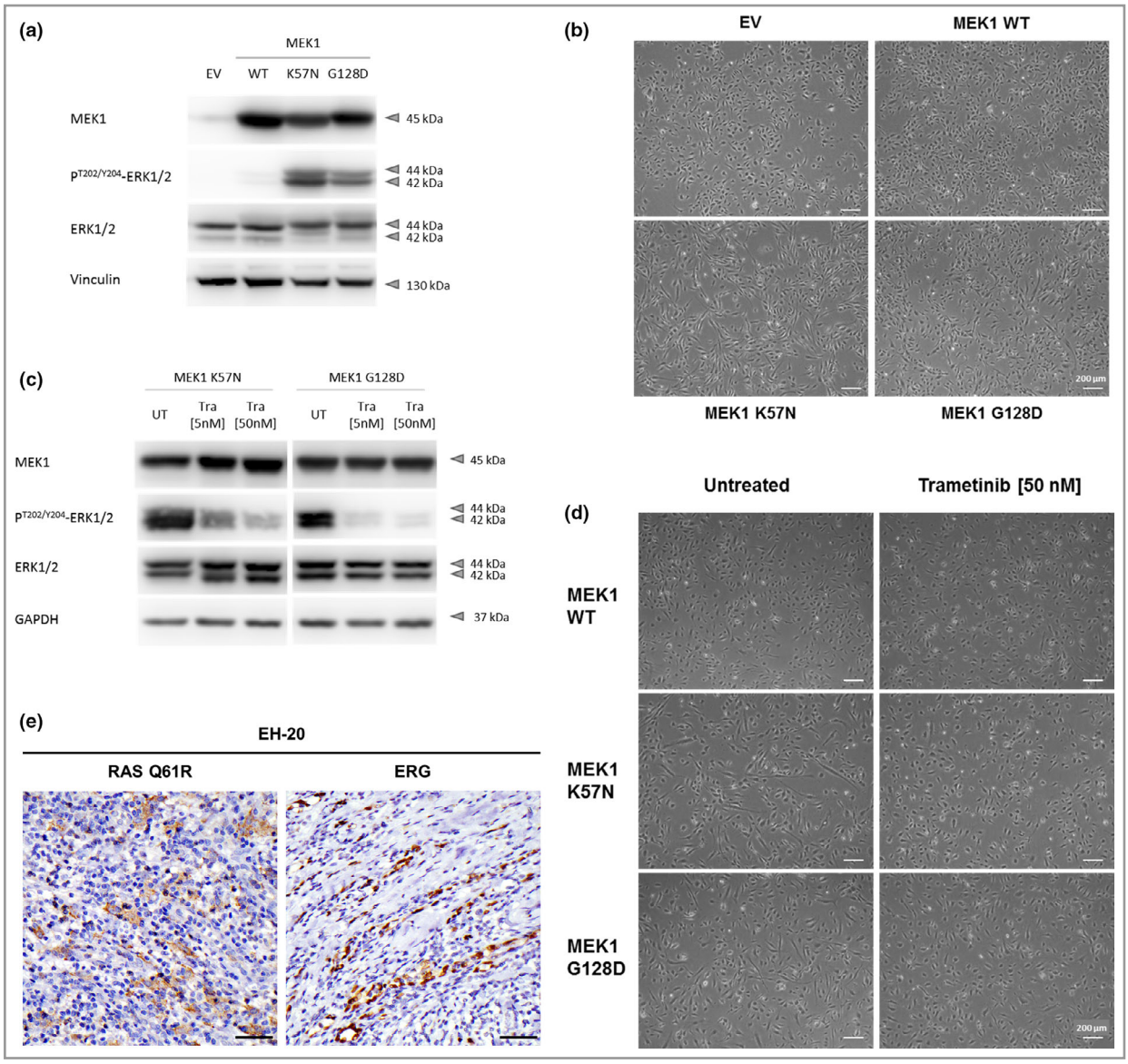

Figure 5 Functional effect of *MAP2K1* variants and presence of mutated KRAS protein in endothelial cells. (a) Western blot analysis of MAPK pathway activation in HUVECs with ectopic expression of wildtype MEK1 (WT), MEK1^{K57N} or MEK1^{G128D} compared with empty vector (EV) transduced control cells. Vinculin was used as loading control. (b) Micrographs taken 10 days after transduction depicting increased cell size and elongated morphology after overexpression of the MEK1 mutants. Scale bars ≙ 200 µm. (c) Western blot analysis of MAPK pathway activation in MEK1^{K57N} or MEK1^{G128D} transduced HUVECs after treatment with the MEK1/2 inhibitor trametinib for 24 h. GAPDH was used as loading control. (d) Corresponding micrographs including MEK^{WT} transduced HUVECs. Scale bars ≙ 200 µm. (e) Immunohistochemical staining of EH-20 tissue specimen for Q61R-mutated KRAS (left) or the endothelial cell marker protein ERG (right). Scale bars ≙ 50 µm. HUVECs, human umbilical vein endothelial cells; MAPK, mitogen-activated protein kinase

an eruption of small independent lesions might indicate that it is rather a late-manifesting vascular malformation than a genuine vascular tumour. Activating, somatic mutations in genes of the MAPK pathway might act as a second hit in cutaneous EH.[38] To clarify if cutaneous EH is a late-manifesting vascular malformation, further comprehensive studies to define the pathogenic events and experimental studies are needed.

Overall, the MAPK pathway seems to play a major role in atypical endothelial growth. For instance, endothelial cells with constitutively activated RAS signalling show increased proliferation as well as migration and acquired a pro-angiogenic phenotype.[39] In this study, we could confirm the activating potential of both detected *MAP2K1* mutations in endothelial cells *in vitro*, by strongly elevated phospho-ERK1/2 levels and increased cell size. MEK1/2 inhibition could reverse these effects *in vitro*. Recently, a small study reported that cobimetinib shows significant clinical activity in histiocytic neoplasms with *MAP2K1* mutations other than those found in cutaneous EH.[40] MEK1 therefore constitutes a potential drug target but clinical evidence is needed to fully understand the druggability of MEK1 in cutaneous EH.

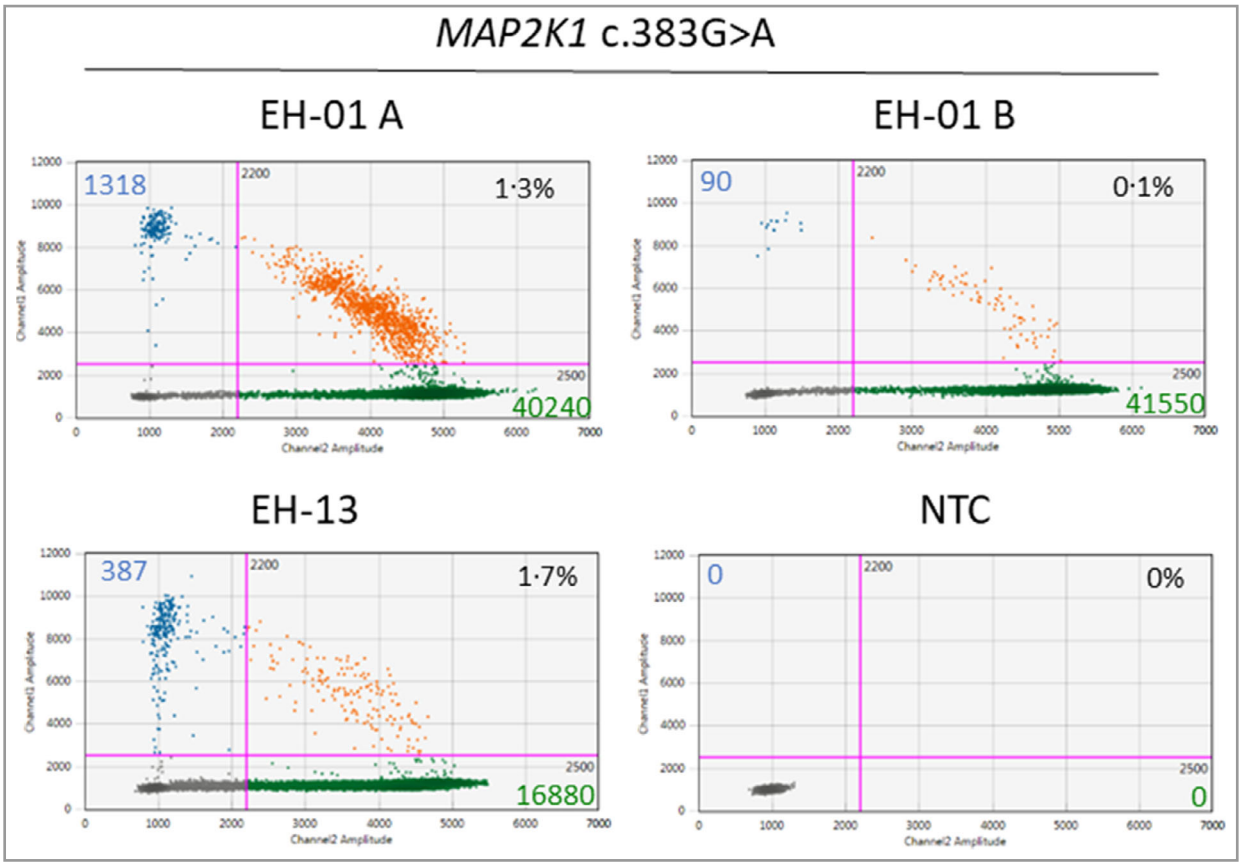

Figure 6 *MAP2K1* c.383G>*A* mutations in epithelioid haemangioma (EH) samples of the index patient detected by droplet digital polymerase chain reaction analysis. Scatter plots depict the droplets containing only the wildtype allele (right lower quadrant), the mutated allele (left upper quadrant) or both alleles (right upper quadrant) in samples of the index patient (EH-01A: endothelia-enriched, EH-01B: lymphocyte-enriched, EH-13: earlier lesion) or in a no template control (NTC) containing no DNA. The total numbers of droplets positive for mutant (blue) or wildtype allele (green) and the resulting mutant allele frequencies (black) are indicated.

In conclusion, our data suggest that a substantial proportion of cutaneous EH is associated with somatic mutations in genes of the MAPK signalling pathway. While this finding does not ultimately clarify if those are causative and whether EH is a benign tumour or rather a vascular malformation, our data indicate that potentially targetable oncogenic events are associated with the development of cutaneous EH.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Table S1 Histological subclassification of cutaneous epithelioid haemangioma.
Table S2 Genes covered by the Oncomine Focus Assay.
Table S3 Multiplex-polymerase chain reaction panel sequencing read statistics.
Table S4 Primers, Probes and gBlocks positive control DNA fragments used for droplet digital polymerase chain reaction.
Table S5 Quantification of human umbilical vein endothelial cell size after ectopic expression of wildtype MEK1 or MEK1 mutants.
Table S6 Quantification of cell size after MEK inhibition in MEK1 transduced human umbilical vein endothelial cells.
Table S7 Somatic variants in the mitogen-activated protein kinase pathway identified with hybridization-based multiplex-polymerase chain reaction panel sequencing.
Table S8 Droplet digital polymerase chain reaction technical details and mutant allele frequencies with 95% confidence intervals.
Figure S1 Representative micrographs of cutaneous epithelioid haemangioma from microdissected areas taken for DNA extraction.
Figure S2 Immunohistochemical staining of a $KRAS^{Q61R}$ mutation-positive colon carcinoma biopsy and of epithelioid haemangioma tissue specimens without detected *KRAS* mutation.
Figure S3 Cell size of human umbilical vein endothelial cells after overexpression of MEK1 mutants and treatment with the MEK inhibitor trametinib.