

# Recurrent Alterations in the MAPK pathway in Sporadic Pyogenic Granuloma of Childhood

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**Pyogenic granuloma is one of the most common vascular tumours. The cause of pyogenic granuloma was previously thought to be an inflammatory reaction with consecutive stimulation of endothelial cell proliferation. However, recent studies suggest that pyogenic granuloma may be driven by constitutive activation of the mitogen-activated protein kinase pathway. The aim of this study was to investigate the molecular profile of sporadic pyogenic granuloma of childhood, using a systematic approach scrutinizing potential aberrations within different oncogenic pathways. Within a retrospective setting pyogenic granuloma of 15 patients was analysed by targeted next generation sequencing using the OncoPrint Focus Assay, which includes genes of key tumorigenic signalling pathways. Activating mutations were found in 4 out of 15 cases (27%). Two *HRAS* hotspot mutations (p.Gly13Arg, p.Ala59Thr), 1 *BRAF* (p.Val600Glu) mutation and a novel, previously not reported, *MAP2K1* hotspot mutation (p.Glu203Lys) were identified. It is notable that all of these genes are involved in constitutive mitogen-activated protein kinase signalling. This study increases the range of underlying genetic alterations in pyogenic granuloma by identifying novel oncogenic mutations in crucial mitogen-activated protein kinase pathway genes. The results provide supporting evidence that activated mitogen-activated protein kinase signalling is a key driver in the pathogenesis of pyogenic granuloma, which might be exploited by targeted treatment approaches for selected cases.**

**Key words:** pyogenic granuloma; targeted sequencing; MAPK pathway.

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Vascular anomalies (vascular tumours and vascular malformations) represent a broad spectrum of disorders with diverse biological behaviour and distinct clinical presentation (1). Evidence to support a more precise classification has grown in recent years, especially based on more sophisticated genetic analyses and, thus, deeper molecular insight.

## SIGNIFICANCE

Pyogenic granuloma is a common vascular tumour. This retrospective study analysed sporadic pyogenic granuloma of 15 children, using targeted next generation sequencing of genes implicated in key tumorigenic signalling pathways. In 27% of analysed cases alterations in genes of the mitogen-activated protein kinase pathway were identified, thereby implying that constitutive mitogen-activated protein kinase signalling plays a critical role in the pathogenesis of sporadic PG. Hence, the previous concept that pyogenic granuloma reflects a reactive endothelial cell proliferation due to inflammatory triggers is revised. Despite somatic alterations in oncogenes, the biological nature of pyogenic granuloma is benign, implying mechanisms such as oncogene-induced senescence.

Pyogenic granuloma (PG) is one of the most common vascular tumours. It shows a benign behaviour and may affect both skin and mucous membranes. Clinically, PG usually presents as a solitary, red, exophytic papule. Iterative bleeding is the most common symptom leading to consultation with a physician. In some cases, satellite lesions are present (agminated PG) or disseminated lesions are rare. PG is commonly located in the head and neck area.

PG was previously thought to be caused by an inflammatory (eponymously “granulomatous”) reaction to an infectious or traumatic event, leading to the term “pyogenic granuloma” (2). However, in recent years, deeper insight into the molecular pathogenesis of PG has been gained. Based on these findings, the previously assumed putative reactive origin of PG has been debated. As a consequence, the term “lobular capillary haemangioma” has been proposed to better classify this vascular tumour. PGs have been observed to arise occasionally in association with vascular malformations, such as a port-wine stain (PWS) (3), but in most cases they occur spontaneously without a syndromic context.

PWS commonly exhibit activating mutations in *GNAQ*, occurring in more than 90% of cases (4). Recently, it has been shown that PG arising within the context of a PWS share similar *GNAQ* mutations with the underlying PWS. However, in PWS-associated PG further somatic single nucleotide variations (SNV) are present, distinguishing both vascular lesions at a molecular level. In a mutually

exclusive pattern both oncogenic *BRAF* mutations (*BRAF* c.1799T>A (p.Val600Glu)) as well as (albeit at lower frequencies) *RAS* mutations (*NRAS* c.182A>G (p.Q61R)) were detected in PWS-associated PG. The underlying PWS revealed wild-type status for both *BRAF* or *NRAS* in all analysed cases. Likewise, sporadic PG of adults and children (occurring without the context of PWS) have been shown to harbour different *BRAF*, *KRAS*, *NRAS* or *HRAS* mutations with variable frequencies within the different studies (5, 6). In line with these molecular data, Pereira et al. demonstrated activation of the mitogen-activated protein kinase (MAPK) pathway on a more functional level by immunohistochemically detecting phosphorylation of the downstream targets ERK1/2 in endothelial cells (7). Taken together, these findings indicate that PG is a true neoplastic condition, which may be driven by constitutive activation of the MAPK pathway on the ground of oncogenic somatic mutations.

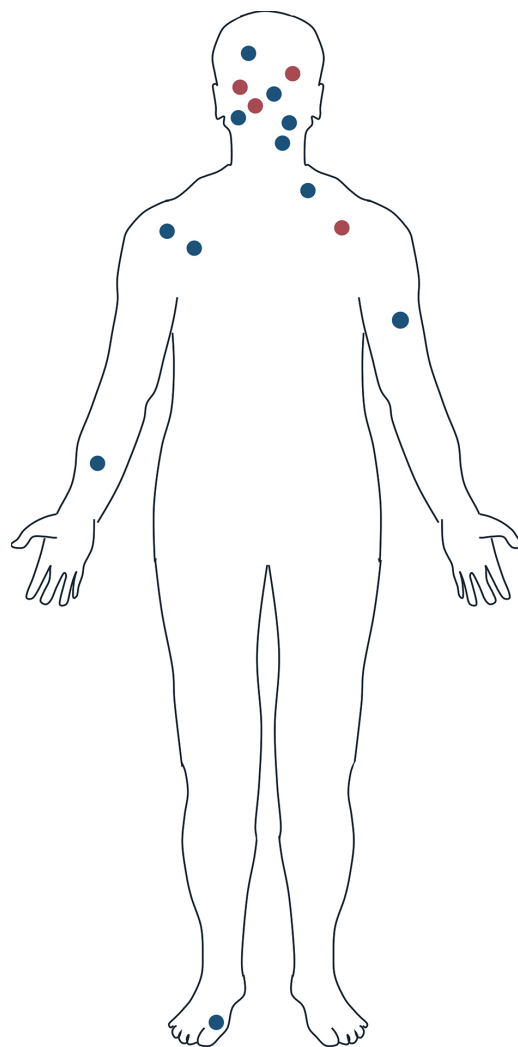
Motivated by these observations this study investigated the molecular profile of sporadic PG of childhood in more detail by taking advantage of targeted next generation sequencing using the OncoPrint Focus Assay (Thermo Fisher Scientific, Darmstadt, Germany). This approach enables investigation of the presence of pathogenic activating mutations within cardinal key oncogenic signalling components including, but not restricted to, the MAPK pathway.

## MATERIALS AND METHODS

### Patient characteristics

Fifteen patients with sporadic PGs, age range 3 months to 16 years, mean age 6.7 years, were retrospectively identified in the clinical and histological files of the Department of Dermatology, University Hospital Würzburg, Germany. All patients were diagnosed with PG in close clinicopathological correlation. The male to female ratio was 3:1. The most frequently affected site was the head/neck area (9 out of 15). All patients underwent surgery (excision, curettage or shave) under local anaesthesia achieving a complete remission. Further patient details are shown in **Table I**. An overview of the anatomical distribution of respective lesions is shown in **Fig. 1**. Representative clinical images are shown in **Fig. 2**. Study approval

was obtained from the ethics committee at the Medical Faculty of the University of Würzburg, Germany, in compliance with the Declaration of Helsinki. Informed written consent was obtained from patients or their parents prior to analysis. Patients/parents also consented to publication of their images.

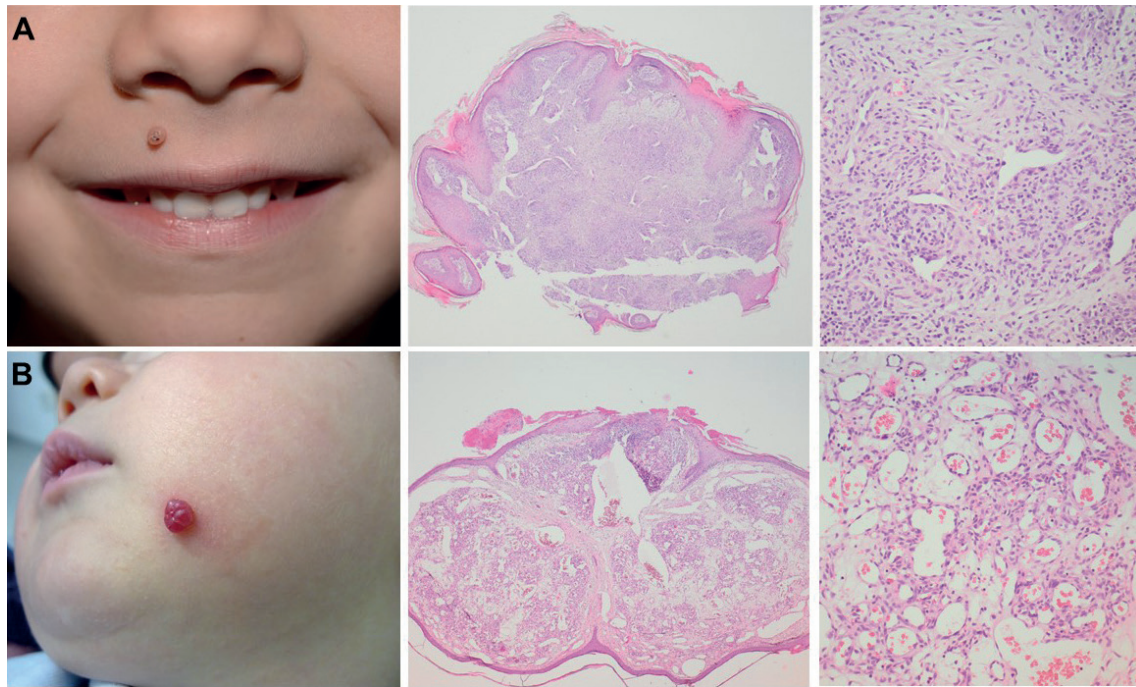


**Fig. 1. Anatomical distribution of pyogenic granuloma (PG).** Out of 15 cases, 4 PG tested positive for *GNAQ*, *BRAF*, *HRAS* or *MAP2K1* mutations (red), while 11 PG showed no evidence of mutations (blue).

**Table I. Patient characteristics and molecular data**

Pat. No.	Sex	Age	Site of presentation	Treatment	Mutation
1	F	9 years	Head (upper lip)	Shave	WT
2	M	2 years	Head (forehead)	Curettage and electrocaustic haemostasis	WT
3	M	12 years	Shoulder	Local excision with primary wound closure	WT
4	M	7 months	Head (nose)	Curettage	WT
5	M	16 years	Shoulder	Local excision with primary wound closure	WT
6	F	11 years	Head (lower lip)	Local excision with primary wound closure	WT
7	M	9 years	Upper arm	Punch excision	WT
8	M	4 years	Head (cheek)	Punch excision	WT
9	M	4 years	Head (upper lip)	Curettage	<i>HRAS</i> c.37G>C, p.Gly13Arg
10	F	12 years	Thoracic	Shave	<i>BRAF</i> c.1799T>A, p.Val600Glu
11	F	7 years	Neck	Punch excision	WT
12	M	3 months	Head (cheek)	Curettage	<i>GNAQ</i> c.548G>A, p.Arg183Gln, <i>HRAS</i> c.175G>A, p.Ala59Thr
13	M	7 years	Right big toe	Shave and electrocaustic haemostasis	WT
14	F	12 years	Head (infraorbital)	Curettage	<i>MAP2K1</i> c.607G>A, p.Glu203Lys
15	M	5 years	Forearm	Local excision with primary wound closure	WT

Summary of clinical characteristics of 15 individuals with sporadic PG in correlation to molecular findings. Abbreviations: F: female; M: male; WT: wild-type sequence.



**Fig. 2. Representative clinical/histological images.** (A) A 4-year-old boy (patient #9) with a skin-coloured, centrally crusted nodule on the right upper lip, tested positive for an *HRAS* c.37G>C mutation (matoxylin and eosin (HE) staining, magnification 20× (left part), magnification 40 x (right part)). Histology shows a lobulated vascular tumor with a reactive inflammatory infiltrate and an adjacent epidermal collarette. (B) A 3-month-old infant (patient #12) with a red, pedunculated, blackberry-like nodule on the left cheek, tested positive for a *GNAQ* c.548G>A and a *HRAS* c.175H>A mutation (HE staining, magnification 20× (left part), magnification 40 x (right part)). Notably, there is no evidence of an underlying vascular malformation/port-wine stain (PWS). On histology, the exophytic tumor is composed of numerous small capillaries with a lobular architecture.

#### DNA extraction

Genomic DNA was extracted from formalin-fixed, paraffin-embedded sections after microdissection, using the Maxwell RSC Blood DNA Kit (Promega GmbH, Walldorf, Germany). After 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 GmbH, Walldorf, Germany). DNA quantity and quality was assessed by quantitative PCR (TaqMan RNase P Detection Reagents Kit, Life Technologies, Darmstadt, Germany).

#### Multiplex PCR-based panel sequencing

DNA libraries were prepared with the DNA part of the Oncomine Focus Assay and the Ion AmpliSeq Library Kit 2.0, according to the manufacturer's recommendations. Subsequently, libraries were templated and enriched with the Ion OneTouch 2 and the Ion One Touch ES automated systems. Sequencing was performed using semiconductor sequencing technology (Ion Genestudio S5, Thermo Fisher Scientific). Data analysis was performed using the Torrent Server Variant Caller (v 5.10) and the Ion Reporter Software (v 5.12) (Thermo Fisher Scientific, Darmstadt, Germany). Samples were sequenced with a mean depth of 2,922×. Data were filtered for non-synonymous, exonic variants, and splice site variants in the flanking regions, showing an allele frequency of more than

2%. Variants in the general reported population with a minor allele frequency of more than 0.5% in 1,000 Genomes and dbSNP were excluded (**Table II**).

## RESULTS

### *Panel sequencing shows recurrent activating mutations in genes of the mitogen-activated protein kinase signalling pathway*

To decipher the pathogenesis of PG and identify crucial molecular driver mechanisms, explorative targeted next generation sequencing was performed using the Oncomine Focus Assay, which addresses activating genetic alterations within key oncogenic signalling pathways. This approach includes > 50 genes coding for different receptor tyrosine kinases and further pivotal components of well-described oncogenic driver pathways, including the phosphatidylinositol 3-kinase (PI3K) and MAPK signalling pathway. Fifteen samples from children and adolescents with sporadic PG were sequenced. The

**Table II. Somatic variants identified by multiplex PCR-based panel sequencing**

Pat. No.	Chr	Start	End	Gene	ExonicFunc.refGene	cDNA	Protein	DP_Tumor	AD_Ref_Tumor	AD_Alt_Tumor	Alt_Tumor (%)
9	chr11	534286	534286	<i>HRAS</i>	Non-synonymous SNV	c.37G>C	p.Gly13Arg	943	858	85	9
10	chr7	140453133	140453133	<i>BRAF</i>	Non-synonymous SNV	c.1799T>A	p.Val600Glu	1983	1778	202	10
12	chr9	80412493	80412493	<i>GNAQ</i>	Non-synonymous SNV	c.548G>A	p.Arg183Gln	2000	1795	205	10
12	chr11	533881	533881	<i>HRAS</i>	Non-synonymous SNV	c.175G>A	p.Ala59Thr	1994	959	1035	52
14	chr15	66774131	66774131	<i>MAP2K1</i>	Non-synonymous SNV	c.607G>A	p.Glu203Lys	2000	1906	94	5



anatomical localization of sporadic PGs with ( $n=4$ ) and without ( $n=11$ ) mutations is shown in Fig. 1. Representative clinical and histological findings of selected mutated cases are shown in Fig. 2. Table I summarizes clinical characteristics of these 15 individuals in correlation to molecular findings.

Within this cohort, 5 mutations were detected in 4 patients (4 out of 15). Two *HRAS* mutations were identified, affecting codons 13 and 59 (p.Gly13Arg, p.Ala59Thr) in 2 patients (patients #9 and #12). Moreover, a *BRAF* V600E (p.Val600Glu) mutation (patient #10) and a *MAP2K1* mutation in codon 203 (p.Glu203Lys) became apparent (patient #14). Of note, patient #12 showed, in addition to a *HRAS* (p.Ala59Thr) mutation, an activating *GNAQ* hotspot mutation (p.Arg183Gln). This patient was a 3-month-old child with a sporadic PG on the cheek, which was not associated with a PWS phenotype (Fig. 2B). No difference was observed on clinical or histological grounds between mutated or unmutated cases, in that all the cases of PG showed complete remission after removal.

## DISCUSSION

In recent years significant progress has been made in elucidating the molecular pathogenesis of vascular malformations and vascular tumours. In various benign and malignant proliferations of endothelial cells several cardinal signalling pathways have been identified as being altered, especially via somatic mutations. Most of these genetic alterations are not subtype-specific. Similar hotspot mutations are widely distributed among different vascular disorders, somewhat irrespective of biological behaviour. Constitutive downstream signalling affects multiple biological functions, such as cellular proliferation, differentiation, apoptosis, metabolism, cytoskeletal arrangement and vasopermeability of endothelial cells (8, 9).

Recent studies suggest that PG is not a reactive process, but a true neoplastic condition possibly driven by constitutive activation of the MAPK pathway due to somatic mutations (10).

Genes of the guanine nucleotide-binding protein subunit alpha q (Gaq) family (*GNAQ*, *GNAI1*, *GNAI4*) represent upstream activators of different pathways, including the phospholipase C (PLC) pathway, the PI3K-Akt-mTOR pathway, and the MAPK signalling cascade. Gaq proteins have been shown to mediate vascular endothelial growth factor- (VEGF-) dependent migration and proliferation of endothelial cells *in vitro* (11). On this basis, Shirley et al. reported that somatic mutations in the *GNAQ* gene (p.Arg183Gln) in PWS blood vessels resulted in activation of downstream MAPK signalling (4). Another study suggested that *GNAI4* and *GNAI1* mutations in kaposiform haemangioendotheliomas and congenital tufted angiomas induce changes in cellular

morphology and growth factor independence via MAPK activation (12). PG arising within the context of a PWS share similar *GNAQ* mutations with the underlying PWS (5) in addition to further driver mutations of the MAPK pathway being exclusively present in PG.

We describe here the novel finding of a *GNAQ* hotspot mutation (p.Arg183Gln) that occurred in addition to an oncogenic *HRAS* mutation (p.Ala59Thr) in sporadic PG not being associated with a PWS phenotype (13, 14). Taken together the previous and current findings raise the hypothesis that, in sporadic PG also, a second hit (in MAPK genes) in addition to a mutation in components of the Gαq family of genes is necessary to drive the full phenotype (5). In fact, in vascular malformations, such as PWS, only slow progression is observed, whereas vascular tumours, such as PG, proliferate rapidly despite analogous activating *GNAQ* mutations (2, 4). This is in line with the fact that cherry haemangioma invariably displays an indolent behaviour despite frequent (>80%) presence of *GNAI4*, *GNAQ* and *GNAI1* mutations without, however, further single nucleotide variations (SNVs) (15). The oncogenic properties of *GNAQ* mutations in combination with further tumour-promoting events are also evidenced in non-vascular tumours, e.g. uveal melanoma, a metastasis-prone, highly malignant tumour exhibiting recurrent SNVs in genes of the G protein family in conjunction with further genetic aberrations (16, 17).

In 27% of the analysed cases in the current study activating mutations in genes of the MAPK pathway were identified, while mutations in other signalling pathways could not be identified. Although oncogenic mutations were identified in different genes, respective constitutive downstream signalling converges within the same MAPK signalling cascade and, thus, finally drives similar functional biological consequences. In order to elucidate whether further oncogenic pathways beyond the MAPK cascade might be involved in the molecular pathogenesis of PG we applied the OncoPrint Focus Assay, which comprises a wide spectrum of different pathway-related genes. However, all of these other genes showed wildtype sequences. This emphasizes that activation of the MAPK pathway at different stages of the signalling cascade represents the major driving force of PG. As this methodological approach focussed only on a selected set of genes, including the most common hotspot mutations in MAPK genes, we cannot exclude that further genetic alterations activating this pathway might be present in the remaining negative cases in the current study cohort, which are missed by the current analysis. It is notable that 1 of the mutations identified herein, i.e. an oncogenic *MAP2K1* mutation (18, 19), has not been described previously in PG, but also fits into the concept of an activated MAPK pathway.

Driver mutations in *RAS* (mainly *KRAS*) are the most common mutations in cancer, occurring in approximately

30% of all cancer types (20). *RAS* mutations are attributed to have a proangiogenic effect on endothelial cells (21). Moreover, *RAF* mutations (particularly in *BRAF*) are present in approximately 8% of all cancer types (22). Although genetic aberrations of these same genes have been shown to orchestrate a malignant phenotype in distinct vascular malignant tumours, such as angiosarcoma (23), albeit at lower frequencies, PG does not develop any malignant properties despite these genetic alterations. The underlying mechanism for this observation is hitherto unknown, but may rely on features of oncogene-induced senescence (24).

To date, no mutations in the MAPK pathway have been detected in either periocular or oral/mucosal PGs. We have now identified an activating *MAP2K1* hotspot mutation in a PG located infra-orbitally. The fact that, altogether, in only 20–30% of cases of PG single nucleotide variations (SNVs) could be detected in different studies, may be due to limited patient number (as was also the case in the current analysis) or the variable methodological approaches including single gene assays (7, 25). Therefore, the current findings highlight the advantage of a high throughput sequencing approach, which allows a sensitive and simultaneous analysis of plenty of relevant driver genes involving various signalling pathways prior to single gene analyses.

To summarize, these data extend the spectrum of somatic mutations in PG. The results of this study strengthen the evidence that constitutive MAPK signalling by activating mutations plays a critical role in the pathogenesis of sporadic PG.

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

*The authors have no conflicts of interest to declare.*

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