Mutational Analysis of Hedgehog Signaling Pathway Genes in Human Malignant Mesothelioma

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Abstract

Background: The Hedgehog (HH) signaling pathway is critical for embryonic development and adult homeostasis. Recent studies have identified regulatory roles for this pathway in certain cancers with mutations in the HH pathway genes. The extent to which mutations of the HH pathway genes are involved in the pathogenesis of malignant mesothelioma (MMe) is unknown.

Methodology/Principal Findings: Real-time PCR analysis of HH pathway genes PTCH1, GLI1 and GLI2 were performed on 7 human MMe cell lines. Exon sequencing of 13 HH pathway genes was also performed in cell lines and human MMe tumors. In silico programs were used to predict the likelihood that an amino-acid substitution would have a functional effect. GLI1, GLI2 and PTCH1 were highly expressed in MMe cells, indicative of active HH signaling. PTCH1, SMO and SUFU mutations were found in 2 of 11 MMe cell lines examined. A non-synonymous missense SUFU mutation (p.T411M) was identified in LO68 cells. In silico characterization of the SUFU mutant suggested that the p.T411M mutation might alter protein function. However, we were unable to demonstrate any functional effect of this mutation on Gli activity. Deletion of exons of the PTCH1 gene was found in JU77 cells, resulting in loss of one of two extracellular loops implicated in HH ligand binding and the intracellular C-terminal domain. A 3-bp insertion (69..70insCTG) in SMO, predicting an additional leucine residue in the signal peptide segment of SMO protein was also identified in LO68 cells and a MMe tumour.

Conclusions/Significance: We identified the first novel mutations in PTCH1, SUFU and SMO associated with MMe. Although HH pathway mutations are relatively rare in MMe, these data suggest a possible role for dysfunctional HH pathway in the pathogenesis of a subgroup of MMe and help rationalize the exploration of HH pathway inhibitors for MMe therapy.

Introduction

Malignant mesothelioma (MMe) is an aggressive, incurable cancer that arises from mesothelial cells that line the serosal cavities of the pleura, peritoneum, pericardium and tunica vaginalis testis. Occupational asbestos exposure is the main risk factor for MMe, accounting for 80% of the cases in men and 40% of the cases in women [1]. The global incidence of MMe has been on the rise since the 1960s and is projected to increase until at least 2050 due to the widespread use of asbestos during the past decades [2–5]. MMe is highly refractory to current treatment modalities including chemotherapy, radiotherapy and surgery, with a short median survival time of less than 12 months after first diagnosis [6]. There is no doubt that successful treatment will require a paradigm shift in how MMe is viewed as a disease. Current research is focusing on the molecular mechanisms underlying MMe development and growth to identify new targets for therapeutic intervention [7].

The Hedgehog (HH) signaling pathway regulates critical aspects of embryonic development as well as adult tissue homeostasis and stem cell maintenance [8–10]. Recently, the HH pathway has been implicated as a major contributor to the growth and maintenance of a variety of human cancers [11]. HH acts as a ligand for the Patched (PTCH) receptor proteins, PTCH1 and PTCH2 [12]. In the absence of the HH ligand (Sonic HH (SHH), Desert HH (DHH) and Indian HH (IHH)), PTCH interaction with Smoothened (SMO) inhibits SMO function [13]. Upon ligand binding, PTCH-mediated repression of SMO is relieved and SMO transduces the signal to a SUFU-GLI complex residing in the cytoplasm, resulting in the release and activation of GLI transcription factors [14]. SUFU is the main repressor of the mammalian HH signaling pathway by sequestering GLI tran-

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scription factors in the cytoplasm and nucleus [15]. This repressor is negatively regulated by serine/threonine kinase 36 (STK36), which in turn promotes activation and nuclear accumulation of GLI [16]. In addition to SUFU, the transmembrane HH-interacting protein (HHIP) was identified as another negative regulator of the HH pathway that acts by sequestering all three HH homologs with similar affinity to that of PTCH1 protein [17]. Recently, KIF7 was identified by sequence comparison as a GLI-interacting protein [18]. KIF7 physically binds to GLI, regulating their stability and degradation and controlled GLI-mediated transcription [19]. There are 3 GLI proteins in vertebrates; GLI1, GLI2 and GLI3, which are capable of either transcriptional activation or repression of cell type-specific HH pathway target genes [20]. A schematic diagram depicting the Hedgehog signaling pathway is shown in Figure 1.

Mutations in PTCH1 have been found in familial basal cell carcinoma (BCC) and medulloblastoma (MB) [21,22]. It was later discovered that sporadic BCCs harbored somatic PTCH1 mutations [23]. PTCH1 mutations have also been found in other sporadic tumors, such as MB [24], skin trichoepitheliomas [25], esophageal squamous cell carcinomas [26], skin squamous cell carcinomas [27], meningiomas [24], breast carcinomas [24] and bladder carcinomas [28], as well as in odontogenic keratocysts [24,27,29–31]. In addition to primary tumors, missense mutations in PTCH1 have been identified in several oral squamous cell carcinoma and colon carcinoma cell lines [24,32].

Clearly mutations in PTCH1 do not account for all cases of familial as well as sporadic BCCs and MBs. Indeed, mutations in several genes in the HH pathway have been found in familial and sporadic BCCs and MBs. In a subset of patients with sporadic BCC, mutations in SMO have been described [33–37] while PTCH2 mutations were detected in some cases of sporadic BCCs and MBs [38]. Unlike PTCH1, PTCH2 is overexpressed in both familial and sporadic BCCs [39] and MBs [40], suggesting that PTCH2 is a direct gene target of HH signaling and that PTCH2 may be negatively regulated by PTCH1 [39]. Loss of PTCH2 has been reported to contribute to enhanced tumorigenesis in PTCH1 haploinsufficient mice [41].

**Figure 1. Hedgehog signaling pathway.** HH ligands (SHH, DHH or IHH) bind to the PTCH receptors (PTCH1 and PTCH2) and relieve the inhibition of SMO. HHIP, a negative inhibitor of HH signaling, can compete with PTCH receptors to bind HH ligand, resulting in the attenuation of HH signaling. SMO then transduces signals through the cytoplasmic SUFU-GLI complex, resulting in the activation and nuclear translocation of the downstream GLI transcription factors (GLI1-3). STK36 further promotes activation and nuclear accumulation of GLI by antagonizing SUFU. KIF7 regulates GLI-mediated transcription both positively and negatively through physical interaction with GLI and regulating the stability and degradation of GLI proteins. doi:10.1371/journal.pone.0066685.g001
Mutations in \textit{SUFU} were detected in sporadic BCCs and seemed to predispose to MB [33,42]. Mutations in \textit{SHH} have also been identified in a number of cancer types, including BCC [43], MB [45], breast carcinoma [43], glioblastoma [44] and lung cancer [45]. Oro and coworkers detected an identical somatic mutation in the putative zinc hydrolase site of \textit{SHH} that caused a histidine to tyrosine substitution at codon 133 of \textit{SHH}. Notably, this mutation occurred in a sporadic BCC, a MB and a breast carcinoma, hinting at the possible involvement of this non synonymous substitution in driving malignancies in these tumor types [43].

To our knowledge, mutations in HH pathway genes have not yet been reported in MMe. In this study, we report identification of a novel multi-exonic deletion in \textit{PTCH1} and an insertion mutation in \textit{SMO} in human MMe, identified via direct sequencing of genes encoding HH signaling pathway proteins in a panel of 7 MMe cell lines. We also identified a point mutation close to the Gli-binding domain of \textit{SUFU} that led to the replacement of threonine for methionine at amino acid position 411. However, Gli1 luciferase reporter assays revealed that this mutation failed to disrupt the inhibitory function of SUFU. We also genotyped a collection of primary tumors for the presence of these mutations and found one tumor sample that carried the \textit{SMO} insertion mutation. In addition, we also detected a number of previously reported single nucleotide polymorphisms (SNPs) in the course of our screen. Six of these sequence variants were predicted to have substantial impact upon the function of the encoded protein.

\section*{Materials and Methods}

\subsection*{Cell Lines and Culture Conditions}

The human MMe cell lines MSTO-211H (CRL-2081), NCI-H28 (CRL-5820), NCI-H226 (CRL-5826), NCI-H2052 (CRL-5915) and NCI-H2452 (CRL-5946) as well as the mouse embryonic fibroblasts C3H/10T1/2 (CCL-226) were obtained from the American Type Culture Collection (ATCC). The 5 MMe cell lines JU77, LO68, NO36, ONE58 and STY51 were kind gifts from Professor Bruce W. Robinson, University of Western Australia, Australia [46] and REN cells were provided by Professor Steve Albelda, University of Pennsylvania, USA. These cell lines were cultured in high glucose Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10\% fetal bovine serum, 4 mM L-glutamine, 100 units/ml penicillin and 100 \mu g/ml streptomycin (all from Invitrogen) in a humidified incubator with 5\% CO$_2$ at 37°C. In addition, normal pericardial mesothelial cells were obtained from patients undergoing thoracic surgery as described previously [47].

\subsection*{Ethics Statement}

Use of the archival tissue blocks in this retrospective study was approved by the Sydney Local Health District Human Research Ethics Committee (Concord Hospital), as part of a larger study to identify prognostic factors in malignant pleural mesothelioma. Consent for the use of these samples was waived by the ethics committee, consistent with the Human Tissue Act (1983) and the Consent for the use of these samples was waived by the ethics committee, consistent with the Human Tissue Act (1983) and the Commonwealth of Australia, 2007). The human MMe cell lines MSTO-211H (CRL-2081), NCI-H28 (CRL-5820), NCI-H226 (CRL-5826), NCI-H2052 (CRL-5915) and NCI-H2452 (CRL-5946) as well as the mouse embryonic fibroblasts C3H/10T1/2 (CCL-226) were obtained from the American Type Culture Collection (ATCC). The 5 MMe cell lines JU77, LO68, NO36, ONE58 and STY51 were kind gifts from Professor Bruce W. Robinson, University of Western Australia, Australia [46] and REN cells were provided by Professor Steve Albelda, University of Pennsylvania, USA. These cell lines were cultured in high glucose Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10\% fetal bovine serum, 4 mM L-glutamine, 100 units/ml penicillin and 100 \mu g/ml streptomycin (all from Invitrogen) in a humidified incubator with 5\% CO$_2$ at 37°C. In addition, normal pericardial mesothelial cells were obtained from patients undergoing thoracic surgery as described previously [47].

\subsection*{Patients and Tumors}

The MPM tumour samples used in this study (Table 1) were part of a series collected from patients who underwent extrapleural pneumonectomy at Royal Prince Alfred or Strathfield Private Hospitals (Sydney, Australia) between 1994 and 2009 [48]. The tumour content in the formalin-fixed paraffin embedded (FFPE) blocks was marked on whole sections to enable enrichment of tumour content via subsequent laser-capture microdissection.

\section*{RNA Isolation, cDNA Synthesis and Quantitative Real-time PCR Analysis (qRT-PCR) of Gene Expression}

Total RNA was extracted from cell lines using an RNaseasy Mini kit (Qiagen). Two micrograms of total RNA was reverse-transcribed into cDNA using random primers (Invitrogen) and Omniscrypt RT kit (Qiagen). TaqMan gene expression assays (Applied Biosystems) were used for quantifying the mRNA expression levels of \textit{GLI1}, \textit{GLI2} and \textit{PTCH1}, \textit{PGK1} was included as the endogenous control. Real-time PCR was carried out on the StepOne Plus Real-Time PCR System (Applied Biosystems) in duplicates. Fold change in gene expression relative to \textit{PGK1} was calculated using the formula as follows:

\begin{equation}
\text{Fold change} = 2^{\Delta Ct}, \text{where } \Delta Ct = Ct(\text{Target}) - Ct(\text{PGK1}).
\end{equation}

\section*{DNA Isolation, PCR Amplification and DNA Sequence Analysis}

Genomic DNA was isolated from cells using the PureLink Genomic DNA kit (Invitrogen) and from FFPE samples using the FFPE DNA minikit (Qiagen), each according to the manufacturer’s instructions. PCR primers that amplify the exons and flanking intronic sequences of 13 HH pathway genes (Table 2) were either obtained from published literature [49,50] or designed using GenBank sequences and the Vector NTI 11.0 software. The primer sequences are listed in Table S1. PCR amplification of HH pathway genes was performed as described previously with slight modifications [51]. PCR was carried out on a iCycler thermal cycler (BioRad) in a 20 \mu l volume containing 10 ng genomic DNA, 1x GoTaq Flexi buffer (Promega), 1.5 mM MgCl$_2$ (Promega), 0.2 mM dNTPs (Invitrogen), 0.5 \mu M primers (Geneworks Pty Ltd), 6\% DMSO (Sigma Aldrich), 1.25 U GoTaq Flexi DNA polymerase (Promega). The optimized PCR conditions for each primer pair are listed in Table S2. PCR products were

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
Sample ID & Subtype & Gender & Age & Survival (months) \\ 
\hline
24 & Epithelioid & M & 53 & 9.56 \\ 
31 & Biphasic & M & 60 & 5.42 \\ 
32 & Epithelioid & M & 58 & 41.4 \\ 
48 & Epithelioid & F & 48 & 44.16 \\ 
50 & Epithelioid & M & 54 & 72.9 \\ 
51 & Biphasic & M & 51 & 3.42 \\ 
52 & Epithelioid & M & 61 & 8.28 \\ 
59 & Epithelioid & M & 52 & 13.73 \\ 
65 & Epithelioid & M & 64 & 6.93 \\ 
72 & Epithelioid & M & 47 & 1.94 \\ 
74 & Epithelioid & M & 57 & 8.34 \\ 
77 & Biphasic & M & 56 & 2.56 \\ 
78 & Epithelioid & F & 59 & 14.52 \\ 
79 & Epithelioid & M & 56 & 10.32 \\ 
\hline
\end{tabular}
\caption{Demographic and clinical characteristics of patients with MMe.}
\end{table}

\begin{footnotesize}
\textit{doi:10.1371/journal.pone.0066685.t001}
\end{footnotesize}
visualized on 1% agarose gels before they were sequenced at the Australian Genome Research Facility (AGRF), Perth, Western Australia, using a Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems) and analyzed on a 3730xl DNA Analyzer (Applied Biosystems). Base calling and quality assessment were carried out using the Sequencing Scanner v1.0 software (Applied Biosystems) while sequence assembly was carried out using the Vector NTI v11.0 software (Invitrogen). All sequence variants found were confirmed by an independent PCR and sequencing reaction to exclude PCR artifacts. Seven mesothelioma cell lines (MSTO-211H, NCI-H29, JU77, LO68, NO36, ONE58 and STY51) were originally exon sequenced with a further 4 cell lines (NCI-H226, NCI-H2052, NCI-H2452 and REN) genotyped for identified mutations.

### PCR Assay for Detection of PTCH1 Exon Deletions

To detect exon deletions of the PTCH1 gene in MMe cell lines, we designed a PCR assay using exonic primers directed against exons 18, 19, 20, 21, 22, and 23 of PTCH1. The PCR condition (Protocol 1) and primer sequences are listed in Tables S2 and S3, respectively. PCR products were visualized on 4% agarose gels.

### In silico Characterization of Polymorphisms in Exons

The web-based programs SIFT [52] and PolyPhen2 [53] were employed to predict the potential effect of non-synonymous amino acid substitutions resulting from the genetic alterations. The default settings were used for all parameters of each program.

### GLI Luciferase Reporter Assay

C3H/10T1/2 cells were plated in triplicates on 24-well plates 24 h before transfection. Cells were cotransfected with 25 ng pRL-TK (Promega), 0.1 μg 12XGLI1 luciferase reporter construct (a kind gift from Professor Rune Toftgaard, Karolinska Institutet), 0.15 μg wild-type GLI1 construct (Origene) and 0.15 μg of the appropriate SUFU construct (wild-type SUFU or SUFU (p.T411M) (Blue Heron Bio) using FuGene 6 transfection reagent (Roche), with a 3:1 ratio (v/w) of FuGene 6 to DNA. Cells were harvested using the Dual-Glo Luciferase assay system (Promega), 48 h after transfection according to the manufacturer’s instruction. Luciferase activity was measured using a Wallac 1420 VICTOR2 multilabel plate reader (Perkin Elmer). All reporter assays were normalized to Renilla luciferase activity. All transfections were repeated in at least two independent experiments, which gave reproducible results.

### Statistical Analysis

Statistical calculations were performed using Graphpad Prism 4.03 software (Graphpad Software, Inc.). Student’s t-test was used to determine the significance of luciferase reporter assays, and p<0.05 was considered significant.

### Results

#### The Canonical HH Signaling Pathway is Active in Human MMe Cell Lines

We used qRT-PCR to demonstrate the mRNA expression of transcription factors GLI1 and GLI2, activators of the canonical HH signaling pathway and a downstream HH target gene PTCH1, in a panel of 7 human MMe cell lines (Figure 2). In contrast, normal mesothelial cells expressed extremely low levels of PTCH1 and GLI2 mRNA while GLI1 was undetectable (Figure 2). These data suggest that the components necessary for active HH signaling are expressed at significantly higher levels in all MMe cell lines analyzed, compared with controls. This is consistent with recent findings of GLI1 and HH interacting protein (HHIP) being elevated 2-6-fold in MMe tissues using qRT-PCR analysis, relative to benign pleural tissue [54]. In a similar study, the mRNA and protein levels of GLI1, GLI2 and the signal transducer SMO were found to be overexpressed in 46 MMe tissues, as analyzed by qRT-PCR and immunohistochemistry [55].

#### PTCH1, SMO and SUFU Mutations in MMe Cell Lines

Through de novo sequencing, all the exons of 13 genes encoding components of the HH signaling pathway were screened for mutations in a panel of 7 MMe cell lines. No mutation was found in the exonic regions of SHH, DHH, HH, HHIP, PTCH1, PTCH2, HHIP, KIF7, GLI1, GLI2 and GLI3 genes. However, one non-synonymous mutation in SUFU was identified (Table 3). This missense mutation, which was found in LO68, involved a C>T transition at nucleotide 1252 in exon 10 of SUFU, which substitutes threonine for methionine at position 411 (p.T411M) (Figure 3A). This mutation was characterized by SIFT [52] and PolyPhen2 [53] and determined to be damaging. We have also cross-checked with 1000 Genomes and confirmed that this insertion is not a germline variant. In addition, as shown in Figure 3B, amino acid sequence alignment from various species revealed that Thr411 of SUFU was evolutionarily highly conserved.

Although no point mutations were identified in the exonic region, homozygous deletion of exons 18, 19, 20, 21, 22 and 23 of PTCH1 in JU77 cell line was observed and had been suspected by the absence of PCR product on agarose gel electrophoresis (Figure 4). Subsequently, this was confirmed by PCR using exonic primers and demonstrated complete absence of PCR amplicon in the cell line (Figure 5). In addition to the identification and characterization of point mutations and exon deletions, a 3 base-pair insertion of CTG between nucleotides 69 and 70 in exon 1 of the SMO gene in LO68 cell line was identified. This 69_70insCTG mutation resulted in an in-frame addition of a leucine residue after amino acid 23 of the SMO protein (p.23L_24GinsL) with unknown functional role (Figure 6). The same in-frame insertion was recently reported in two out of 39 gastric tumors [56].

### Hedgehog Pathway Gene Variants in MMe Cell Lines

Screening all the exons of each of the 13 HH pathway genes revealed a total of 35 SNPs using our panel of 7 MMe cell lines

### Table 2. Genes sequenced in this study.

| Gene Symbol | Gene name                        | NCBI Gene ID |
|-------------|----------------------------------|--------------|
| GLI1        | GLI family zinc finger 1         | 2735         |
| GLI2        | GLI family zinc finger 2         | 2736         |
| GLI3        | GLI family zinc finger 3         | 2737         |
| HH1         | indian hedgehog                  | 3549         |
| PTCH1       | patched 1                        | 5727         |
| SHH         | sonic hedgehog                   | 6469         |
| SMO         | smoothened, frizzled family receptor | 6608        |
| PTCH2       | patched 2                        | 8643         |
| STK36       | serine/threonine kinase 36       | 27148        |
| DHH         | desert hedgehog                  | 50846        |
| SUFU        | suppressor of fused homolog (Drosophila) | 51684 |
| HHIP        | hedgehog interacting protein     | 64399        |
| KIF7        | kinesin family member 7          | 374654       |

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Hedgehog Pathway Gene Variants in MMe Cell Lines

Screening all the exons of each of the 13 HH pathway genes revealed a total of 35 SNPs using our panel of 7 MMe cell lines
Examination of the NCBI dbSNP database revealed that all the SNPs were previously reported variations. No polymorphism was found in the exonic regions of DHH and SHH genes. Among the 35 SNPs detected, all were located in the coding regions and 16 SNPs would cause substitution of an amino acid (Table 4). The most frequent SNPs include: (i) GLI2 rs2592595, (ii) GLI2 rs3738880, (iii) GLI2 rs10167980, (iv) GLI2 rs12711538, (v) KIF7 rs3803531, (vi) KIF7 rs8037349, (vii) KIF7 rs8179066 and SMO rs2228617, which were identified in all 7 cell lines. In silico analysis of potential functional impact of the 16 nonsynonymous SNPs were characterized by the SIFT and PolyPhen2 programs. There was an overlap between SIFT and PolyPhen2

Figure 2. Relative expression (expressed in $\Delta Ct$) of the HH pathway genes. For each gene, the relative expression of mRNA was normalized to an endogenous PGK1 control. Values represent the mean ± S.E.M. of three independent experiments each performed in duplicates. A, GLI1, B, GLI2 and C, PTCH1 in 7 MMe cell lines and 2 primary cultures of normal mesothelial cells. doi:10.1371/journal.pone.0066685.g002

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predictions: only one SNP (KIF7 rs8179065) was predicted to be a non-deleterious substitution by both SIFT and PolyPhen2 (Table 4). The remaining five non-synonymous SNPs (PTCH1 rs357564, STK36 rs1344642 and rs1863704, KIF7 rs138354681 and GLI1 rs2228224) were predicted by PolyPhen2 as damaging but benign by SIFT (Table 4).

PTCH1, SMO and SUFU Mutations in FFPE MMe Tumors
To validate the mutations that were identified in the cell lines, we analyzed DNA from micro-dissected FFPE tumors from 14 patients with MMe for PTCH1 exonic deletions, SMO insertion and SUFU point mutation. Sequencing results showed that one patient had a tumor harboring the CTG insertion in the SMO gene. The patient was a male Caucasian who survived for 3.4 months after being diagnosed at age 51 years with biphasic MMe. As blood was not collected from the patient, the germline vs. somatic origin of the mutation could not be determined. Lastly, we did not detect the presence of the PTCH1 exon deletions or the SUFU mutation in the patient cohort.

Functional Characterization of SUFU Mutant
To corroborate the SIFT and PolyPhen2 predictions that the p.T411M mutation affects SUFU function, we transiently transfected C3H10T1/2 cells with a Gli1 luciferase reporter construct and expression constructs for wild-type Gli1, wild-type SUFU and the SUFU p.T411M mutant. Transfection of wild-type Gli1 resulted in increased Gli1 reporter activity whereas cotransfection of wild-type SUFU inhibited Gli1-induced reporter activity. However, the SUFU mutant failed to suppress Gli1 reporter activity (Figure 7).

Table 3. Mutations identified in MMe cell lines.

| Type of alteration | Gene | Exon | Region | Nucleotide change | Amino acid change | SIFT | PolyPhen2 | Cell line |
|--------------------|------|------|--------|-------------------|------------------|------|-----------|----------|
| Point mutation     | SUFU | 10   | Coding | 1232 C>T         | T411M            | Damaging | Possibly damaging | LO68     |
| Deletion           | PTCH1| 18   | Coding | –                | –                | Unknown | Unknown | JU77     |
|                   | PTCH1| 19   | Coding | –                | –                | Unknown | Unknown | JU77     |
|                   | PTCH1| 20   | Coding | –                | –                | Unknown | Unknown | JU77     |
|                   | PTCH1| 21   | Coding | –                | –                | Unknown | Unknown | JU77     |
|                   | PTCH1| 22   | Coding | –                | –                | Unknown | Unknown | JU77     |
|                   | PTCH1| 23   | Coding | –                | –                | Unknown | Unknown | JU77     |
| Insertion          | SMO  | 1    | Coding | 69_70insCTG      | 23L_24GinsL      | Unknown | Unknown | LO68     |

Figure 3. Identification of a SUFU<sup>T411M</sup> mutation in LO68 cell line. A, Electropherogram of SUFU gene. Left, the sequencing result of MSTO-21H cell line, showing the wildtype SUFU gene. Right, the sequencing result of LO68 carrying the mutant allele. B, Amino acid sequence alignment of SUFU from various species. Shown in the red box is the T411 residue that is evolutionarily conserved among these different species. Numbers indicate the position of amino acid residue with the start codon (methionine) as number 1.

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Discussion

We examined the expression profiles of the HH gene components in MMe cell lines and cultured primary mesothelial cells. We found that the HH pathway components GLI1, GLI2 and PTCH1 are expressed at high levels in MMe cell lines compared to normal mesothelial cells. This suggests that HH signaling is active...
in Me, which corroborated findings from an earlier study [54,55].

Aberrant activation of HH signaling in human cancers could result from genetic alterations in pathway components, including PTCH1, SMO, SUFU and GLI1 [37,42,57,58]. Utilizing de novo sequencing of PCR-amplified DNA fragments, we present for the first time a mutational analysis of 13 genes encoding components of the HH signaling pathway in 7 Me cell lines. The rationale for screening HH pathway genes for genetic alterations is based on several observations: 1) this pathway plays a critical role in development and growth, 2) high frequency of mutations in the HH pathway genes screened to date, and 3) the HH pathway components were found to be overexpressed in primary Me tumors and cell lines [54,55].

In the present study, only three mutations were found in 3 out of 13 genes screened and they were detected in only two cell lines (Table 3). The first mutation that we have identified is a novel multiple-exon deletion in the PTCH1 gene in JU77 cell line, whereby exons 18–23 were deleted. This mutation would result in a putative truncated PTCH1 protein, in which one of two extracellular loops and the cytoplasmic C-terminal domain are lost, or could undergo nonsense-mediated mRNA decay. However, we did not detect this multi-exonic deletion in our cohort of tumor samples from patients with Me, but this may be due to the small sample size of the patient cohort.

The tumor suppressor gene PTCH1 was cloned in 1996 and subsequently shown to be involved in repression of the HH pathway [13,21,22]. As expected for a classical tumor suppressor gene, PTCH1 was found to be mutated in Gorlin syndrome and many other cancers [59]. Previously published studies on mutations in PTCH1 have found more than 300 mutations, most of which appeared to be clustered in the large extracellular and intracellular loops of the PTCH1 protein [59,60]. Although the functional impact of the deletion of exons 18–23 in PTCH1 has yet to be elucidated, the key role of the extracellular loops in mediating HH ligand binding and the role of the cytoplasmic C-terminal domain in mediating subcellular localization and turnover of PTCH1 as well as inhibition of HH gene targets suggest that the receptor lacking exons 18–23 may lead to an aberrantly activated HH pathway [61–63]. Recent studies also suggest that the C-terminal fragment of PTCH1 could be localized to the nuclear region of the cell where it represses the transcriptional activity of Gli1, even though the canonical view of PTCH1 as a transmembrane protein is deeply entrenched in the literature [64]. In addition to the exonic deletion, we found a SNP (c.3944C>T) in exon 23 of PTCH1, which was also reported in cancers of the skin, bone and vulva [32,65–68]. This non-synonymous SNP leads to substitution of leucine for proline at position 1315 in the C-terminal domain of the PTCH1. Modeling analysis suggested that the Leu1315 substitution might alter the secondary structure of PTCH1 resulting in constitutive pathway activation [69]. Intriguingly, this biallelic SNP has recently been associated with a higher risk for development of breast and nonmelanoma skin cancers [69–71]. The significance of this SNP with respect to etiopathogenesis of Me, however, requires further study.

We also detected a SMO mutation in one Me cell line which was also present in a tumor sample. SMO acts as a signal...
transducer of the HH pathway, mediating communication between transmembrane PTCH1 receptor and transcriptional activators GLI1 and GLI2. Xie et al. found activating somatic mutations in SMO in 3 out of 47 (6.4%) patients with sporadic BCC [37]. Xie et al. went on further to show that overexpression of these mutant SMO proteins in mouse skin produced BCC-like tumors [37]. In this study, the identified mutation, 23L_24GinsL, lies in the signal peptide region of SMO and has been detected in two cases of human gastric cancer {Wang, 2013 #657}. This mutation might affect the processing of the SMO precursor and in

| Gene  | Exon | Region | Nucleotide change | Amino acid change | dbSNP ID | SIFT     | PolyPhen2 | Cell line                          |
|-------|------|--------|-------------------|-------------------|----------|----------|-----------|-----------------------------------|
| GLI1  | 5    | Coding | 576 G>A           | E192E             | rs2228225 | Tolerated | Benign    | JU77, LO68, NO36, STY51           |
| GLI1  | 11   | Coding | 2798 G>A          | G933D             | rs2228224 | Tolerated | Damaging  | JU77, LO68, NO36, STY51           |
| GLI1  | 11   | Coding | 3298 G>C          | E1100Q            | rs2228226 | Tolerated | Benign    | JU77, LO68, NO36, STY51           |
| GLI2  | 5    | Coding | 801 G>A           | S267S             | rs2592595 | Tolerated | Benign    | MSTO-211H, NCI-H28, JU77, LO68, NO36, ONE58, STY51 |
| GLI2  | 13   | Coding | 3466 G>T          | A1156S            | rs3738880 | Tolerated | Benign    | MSTO-211H, NCI-H28, JU77, LO68, NO36, ONE58, STY51 |
| GLI2  | 13   | Coding | 3916 G>A          | D1306N            | rs12711538| Tolerated | Benign    | MSTO-211H, NCI-H28, JU77, LO68, NO36, ONE58, STY51 |
| GLI2  | 13   | Coding | 3939 A>G          | P1313P            | rs10167980| Tolerated | Benign    | MSTO-211H, NCI-H28, JU77, LO68, NO36, ONE58, STY51 |
| GLI3  | 4    | Coding | 547 A>G           | T183A             | rs846266  | Tolerated | Benign    | MSTO-211H, NCI-H28, LO68, ONE58, STY51 |
| GLI3  | 14   | Coding | 2993 C>T          | P998L             | rs929387  | Tolerated | Benign    | JU77, NO36, ONE58                 |
| HHRP  | 13   | Coding | 2058 T>C          | I686I             | rs11727676| Tolerated | Benign    | MSTO-211H, NCI-H28                |
| HH    | 3    | Coding | 600 G>A           | T200T             | rs3731878 | Tolerated | Benign    | STY51                             |
| HH    | 3    | Coding | 753 T>C           | P251P             | rs3731881 | Tolerated | Benign    | MSTO-211H, NCI-H28, JU77, LO68, NO36, ONE58, STY51 |
| HH    | 3    | Coding | 1128 T>C          | T376T             | rs394452  | Tolerated | Benign    | MSTO-211H, NCI-H28, JU77, LO68, NO36, ONE58, STY51 |
| KIF7  | 1    | Coding | 154 G>A           | D52N              | rs8179066 | Tolerated | Benign    | MSTO-211H, NCI-H28, LO68          |
| KIF7  | 1    | Coding | 195 G>C           | A65A              | rs8179066 | Tolerated | Benign    | MSTO-211H, NCI-H28, JU77, LO68, NO36, ONE58, STY51 |
| KIF7  | 4    | Coding | 1102 A>G          | T368A             | rs8037349 | Tolerated | Benign    | MSTO-211H, NCI-H28, JU77, LO68, NO36, ONE58, STY51 |
| KIF7  | 11   | Coding | 2501 A>G          | Q834R             | rs13835468| Tolerated | Damaging  | ONE58                             |
| KIF7  | 12   | Coding | 2658 A>C          | A886A             | rs3803531 | Tolerated | Benign    | MSTO-211H, NCI-H28, JU77, LO68, NO36, ONE58, STY51 |
| KIF7  | 13   | Coding | 2873 G>T          | S958I             | rs3803530 | Tolerated | Benign    | JU77, NO36, ONE58, STY51          |
| KIF7  | 14   | Coding | 3013 G>A          | G1005R            | rs12900805| Tolerated | Benign    | JU77, ONE58, STY51               |
| KIF7  | 14   | Coding | 3048 G>A          | S1016G            | rs9672266 | Tolerated | Benign    | JU77, ONE58, STY51               |
| PTCH1  | 23   | Coding | 3944 C>T          | P1315L            | rs357564  | Tolerated | Damaging  | MSTO-211H, NCI-H28, ONE58, STY51 |
| PTCH2  | 2    | Coding | 90 G>A            | L30L              | rs45573433| Tolerated | Benign    | MSTO-211H, NCI-H28               |
| PTCH2  | 8    | Coding | 1080 G>T          | V360V             | rs11573579| Tolerated | Benign    | STY51                             |
| PTCH2  | 14   | Coding | 1821 A>G          | E607E             | rs2295997 | Tolerated | Benign    | NO36                              |
| PTCH2  | 14   | Coding | 2055 T>C          | A685A             | rs7525308 | Tolerated | Benign    | JU77, NO36                        |
| PTCH2  | 16   | Coding | 2487 C>T          | D829O             | rs2295996 | Tolerated | Benign    | JU77                              |
| SMO   | 1    | Coding | 74 A>G            | D25G              | rs14304185| Tolerated | Unknown   | MSTO-211H, NCI-H28, LO68, NO36    |
| SMO   | 2    | Coding | 384 C>T           | A128A             | rs45571737| Tolerated | Benign    | STY51                             |
| SMO   | 4    | Coding | 808 G>A           | V270I             | rs111694017| Tolerated | Benign    | STY51                             |
| SMO   | 4    | Coding | 852 G>A           | Q284Q             | rs4545295 | Tolerated | Benign    | ONE58                             |
| SMO   | 6    | Coding | 1164 G>C          | G388G             | rs2228617 | Tolerated | Benign    | MSTO-211H, NCI-H28, JU77, LO68, NO36, ONE58, STY51 |
| STK36  | 13   | Coding | 1748 G>A          | R583Q             | rs1344642 | Tolerated | Damaging  | JU77                              |
| STK36  | 24   | Coding | 3008 G>A          | G1003D            | rs1863704 | Tolerated | Damaging  | JU77                              |
| SUFU   | 11   | Coding | 1299 T>C          | I433I             | rs17114803| Tolerated | Benign    | NO36                              |

Table 4. SNPs identified in MMe cell lines.

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Figure 7. p.T411M mutation fails to disrupt the inhibitory function of SUFU. C3H10T1/2 cells were transfected with Gliluciferase reporter construct with Renilla luciferase plasmid for 48 h before they were harvested for luciferase activity determination. Values represent the mean ± S.E.M. of a representative experiment performed in triplicates.
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turn potentially interfere with protein targeting to the cell membrane.

One of the most intriguing results of this analysis is the identification of a mutation in SUFU. SUFU was originally identified as a negative regulator of the HH pathway in early embryonic development [72]. Taylor et al. first implicated SUFU in the tumorigenesis of childhood MB in 2002 [73]. They identified germline and somatic SUFU nonsense mutations in 3.7% (4 of 46) of desmoplastic MBs. Subsequently, SUFU mutations were found in 4.8% (2 of 42) and 2.5% (2 of 83) of sporadic BCCs and MBs, respectively [33,74]. We found a missense mutation affecting Thr411 in SUFU in one of the 11 cell lines. Importantly, the same mutation was recently detected in a patient with colorectal cancer as part of the Cancer Genome Atlas [75], supporting a role of this mutation in tumorigenesis. This mutation was predicted by two web-based programs, SIFT and PolyPhen2, that predict the potential functional impact of altered amino acid sequences on encoded protein function, to result in a major change in amino acid class (from small, polar to large, hydrophobic) in the N-terminal region located close to the GLI1 binding domain, thereby implying a negative impact on protein function. SUFU acts as a classic tumor suppressor gene, with mutations leading to the inability of SUFU to transport GLI1 out of the nucleus to the cytoplasm, thereby resulting in aberrant activation of HH signaling in MB [73]. However, functional characterization showed that the p.T411M mutation does not alter the negative regulatory function of SUFU in a Gliluciferase reporter assay.

In addition to PTCH1, SUFU and SMO, we screened our MMe cell line panel for mutations in 9 other HH pathway genes PTCH2, DHH, IHH, SHH, GLI1, GLI2, GLI3, KIF7, STK36 and HHIP. We found no evidence for mutations in any of these genes in our cell lines. However, we did find a number of previously reported SNPs, of which 16 were shown to result in non-synonymous codon substitution. To explore the functional significance of these SNPs, all those that are non-synonymous were analyzed by SIFT and PolyPhen2. There was considerable difference between the predictions from different algorithms: a KIF7 SNP rs8179065 was predicted by SIFT to have altered protein function whereas the PolyPhen2 program predicted the PTCH1 SNP rs357564, STK36 SNPs rs1344642 and rs1863704, KIF7 SNP rs130354601 and GLI1 SNP rs2229224 to be deleterious amino acid substitution. Algorithmic differences aside, the real functional significance of these non-synonymous SNPs on HH signaling needs to be validated by biochemical studies.

Recently, SNPs in SHH, GLI2 and GLI3 genes have been reported to be associated with clinical outcome of trans-urethral resection and Bacillus Calmette-Guerin intravesical therapy for non-muscle-invasive bladder cancer patients (NMIBC) [76]. The GLI2 SNP rs11685068 and SHH SNP rs1233560 demonstrated significant associations with recurrence in NMIBC patients who received trans-urethral resection. On the other hand, the NMIBC patients receiving Bacillus Calmette-Guerin treatment who carried the GLI3 SNPs rs3801192 and rs6463089 were found to have a higher cancer recurrence rate and shorter recurrence-free survival time compared to those carrying the wildtype genotype. In a separate genome-wide association study, common variants in the SHH, BTRC and HHIP genes have been associated with the risk of pancreatic cancer [77]. These studies demonstrated a link between common genetic variations in the Hedgehog pathway and cancer risk, thus highlighting the importance of examining the association between SNPs in HH pathway genes and the risk of MMe.

Clearly, the frequency of mutations in key HH pathway genes in MMe is less than in BCC and MB where the mutations lead to hyperactivation of the pathway. However, the importance of these and other unidentified mutations in HH pathway genes in the pathogenesis of a subset of MMe tumors cannot be discounted and needs further investigation in a larger sample set.

In summary, we report mutations in SMO and SUFU and a novel multi-exonic deletion in PTCH1 in MMe cell lines and tumors. Our data suggest that unlike BCC, MB and rhabdomyosarcoma in Gorlin syndrome, aberrant activation of HH signaling in MMe is unlikely to be driven by mutations in the majority of tumors but instead activated through autocrine signaling as suggested by Shi et al. [54] This pathway may represent a novel therapeutic target in MMe for the recently developed HH pathway inhibitors.

Supporting Information

Table S1 List of primers used for the PCR amplification and sequencing of Hedgehog pathway genes. (XLSX)

Table S2 PCR conditions used in this study. (XLSX)

Table S3 List of primers used for the detection of PTCH1 exon deletions. (XLSX)

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Author Contributions

Conceived and designed the experiments: CBL SEM. Performed the experiments: CBL HMC. Analyzed the data: CBL SEM CMP SB DNW PJT. Contributed reagents/materials/analysis tools: YYC SK GR PJT. Wrote the paper: CBL SEM.
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