SHORT COMMUNICATION

***TP53*, *MSH4*, and *LATS1* Germline Mutations in a Family with Clustering of Nervous System Tumors**

Young-Ho Kim,^{*†} Takashi Ohta,^{*‡} Ji Eun Oh,^{*‡} Florence Le Calvez-Kelm,[‡] James McKay,[‡] Catherine Voegelé,[‡] Geoffroy Durand,[‡] Michel Mittelbronn,^{§¶||} Paul Kleihues,^{**} Werner Paulus,^{††} and Hiroko Ohgaki^{*}

From the Section of Molecular Pathology^{*} and Group of Genetic Cancer Susceptibility,[‡] International Agency for Research on Cancer, Lyon, France; the Translational Epidemiology Research Branch,[†] National Cancer Center, Goyang, Republic of Korea; the Institute of Neurology (Edinger Institute),[§] Johann Wolfgang Goethe University Frankfurt, Frankfurt/Main, Germany; the German Cancer Consortium (DKTK),[¶] Heidelberg, Germany; the German Cancer Research Center (DKFZ),^{||} Heidelberg, Germany; the Medical Faculty,^{**} University of Zürich, Zürich, Switzerland; and the Institute of Neuropathology,^{††} University Hospital Münster, Münster, Germany

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Address correspondence to
Hiroko Ohgaki, Ph.D., Molecular Pathology Section, International Agency for Research on Cancer, 150 Cours Albert Thomas, 69372 Lyon, France. E-mail: ohgaki@iarc.fr.

Exome DNA sequencing of blood samples from a Li-Fraumeni family with a *TP53* germline mutation (codon 236 deletion) and multiple nervous system tumors revealed additional germline mutations. Missense mutations in the *MSH4* DNA repair gene (c.2480T>A; p.I827N) were detected in three patients with gliomas (two anaplastic astrocytomas, two glioblastomas). Two family members without a *TP53* germline mutation who developed peripheral schwannomas also carried the *MSH4* germline mutation, and in addition, a germline mutation of the *LATS1* gene (c.286C>T; p.R96W). *LATS1* is a downstream mediator of the *NF2*, but has not previously been found to be related to schwannomas. We therefore screened the entire coding sequence of the *LATS1* gene in 65 sporadic schwannomas, 12 neurofibroma/schwannoma hybrid tumors, and 4 cases of schwannomatosis. We only found a single base deletion at codon 827 (exon 5) in a spinal schwannoma, leading to a stop at codon 835 (c.2480delG; p.*R827Kfs*8). Mutational loss of *LATS1* function may thus play a role in some inherited schwannomas, but only exceptionally in sporadic schwannomas. This is the first study reporting a germline *MSH4* mutation. Since it was present in all patients, it may have contributed to the subsequent acquisition of *TP53* and *LATS1* germline mutations. (*Am J Pathol* 2014, 184: 2374–2381; <http://dx.doi.org/10.1016/j.ajpath.2014.05.017>)

Li-Fraumeni syndrome is an autosomal dominant disorder characterized by multiple neoplasms in children and young adults, with a predominance of soft tissue sarcomas, osteosarcomas, breast cancer, brain tumors, leukemia, and adrenocortical carcinoma.^{1,2} Brain tumors are the fourth most frequent tumors and account for approximately 15% of all neoplasms associated with *TP53* germline mutations.¹ Although the mean number of brain tumors per affected family is 0.3, some families show a remarkable clustering of central nervous system neoplasms.¹ Brain tumors are more likely to be associated with missense mutations located on the DNA-binding surfaces of the p53 protein that make contact with the minor groove of DNA.³ Familial clustering may also be due to gene–environment interactions, eg, exposure of families to similar environmental carcinogens or lifestyle factors, which has been

suggested for stomach and breast cancer in Li-Fraumeni families.² However, environmental factors operative in the development of brain tumors have not been identified, except for therapeutic X-irradiation, particularly in children with acute lymphoblastic leukemia.⁴ Another possibility is the presence of multiple germline mutations, which may cooperate toward an increased susceptibility of developing of nervous system tumors. This mechanism may apply to the family reported in this study, which carries germline mutations in three apparently unrelated genes, causing inherited gliomas (*TP53* and *MSH4*) and schwannomas (*LATS1* and *MSH4*).

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Materials and Methods

Family History and Sporadic Schwannoma Samples

The studied family has previously been reported to carry a *TP53* germline mutation (codon 236 deletion) and shows clustering of nervous system tumors.⁵ Later, two family members (patients F and G), who are not carriers of a *TP53* germline mutation, developed schwannomas (Figure 1).⁶ Patient F developed an intraspinal schwannoma at L1/L2 at 39 years of age, and patient G was diagnosed with a peripheral schwannoma at the ischiadic nerve at 43 years of age. Single-strand conformation polymorphism followed by direct sequencing of all *NF2* exons revealed a somatic *NF2* mutation (20-bp insertion 5'-TTTGGTGTGCCGACTC-3' starting from the nucleotide 41264 in exon 2) in the schwannoma of patient G, but not in that of patient F (unpublished data).

We obtained 65 sporadic schwannomas (36 cases located at spinal nerves and 29 cases at peripheral nerves), 12 hybrid neurofibroma/schwannoma tumors, and 4 cases of schwannomatosis from the Institute of Neuropathology, University Hospital Münster (Münster, Germany) and the Institute of Neurology, Goethe-University (Frankfurt, Germany). This study was approved by the International Agency for Research on Cancer ethical committee.

DNA Extraction

DNA was extracted from peripheral blood of patients F and G, as well as from blood samples of 14 unrelated healthy individuals, using the QIAamp DNA Mini Kit (Qiagen, Courtaboeuf, France). Quantification and quality control of DNA was determined by spectrophotometry (NanoDrop

Technologies, Wilmington, DE) and analysis with the 2100 Bioanalyzer (Agilent Technologies, Chesham, UK). DNA was also extracted from paraffin sections of tumor tissues, as previously described.⁷

Preparation of Library and Exome Sequencing

Genomic DNA (1.5 µg) extracted from blood samples of patients F and G was used for whole-exome sequencing library preparation according to the SOLiD Fragment Library preparation protocol (PN4460960 Rev. A; Thermo Fisher Scientific, Waltham, MA) with some modifications. The protocol was adapted so that all reagent volumes were divided in half. Briefly, DNA was sonicated into small fragments with a mean of 160 bp in 60 µL shearing mix containing shearing buffer and low TE buffer using the Covaris S220 Focused-Ultrasonicator system (Covaris, Woburn, MA). Sheared products (60 µL) were then end polished by adding 40 µL of the master mix and then size selected using Agencourt AMPure XP magnetic beads (Beckman Coulter, Brea, CA) to specifically retain DNA fragments between 100 bp and 250 bp. A 3'-dA tail was added to size-selected DNAs, and barcoded adaptors were then ligated before being purified using the Agencourt AMPure XP magnetic beads. Purified ligated products were then amplified with six cycles of PCR and subsequently purified using the Agencourt AMPure XP magnetic beads. Quality controls included quantitation of DNA after size selection, ligation of adaptors, and amplification of DNAs using the Qubit dsDNA HS Assay Kit and the Qubit 2.0 fluorometer (both from Thermo Fisher Scientific), and DNA profiling of the DNAs at the same steps using the Agilent High Sensitivity DNA Kit and the Agilent Technologies 2100 Bioanalyzer.

Purified barcoded and ligated DNA product (62.5 ng of each) were pooled, and exome capture was performed according to the Life Technologies' SOLiD TargetSeq Exome Enrichment System protocol (PN MAN0004396) and TargetSeq Exome Enrichment Kit (both Thermo Fisher Scientific), which is designed to target a 37.3-Mb exomic sequence representing >98% VEGA (<http://vega.sanger.ac.uk/index.html>, last accessed July 28, 2014), CCDS coding regions (<http://www.ncbi.nlm.nih.gov/CCDS/CcDSBrowse.cgi>, last accessed July 28, 2014), and RefSeq exons. Then 125 ng of the pooled barcoded library was hybridized to 2.5 µL of TargetSeq Exome probe pool and buffers at 47°C for 72 hours; probe-hybridized DNA was then washed and recovered using Dynabeads M-270 Streptavidin beads (Invitrogen) in 15 µL of nuclease-free water. This pool was then amplified with 10 cycles of PCR and purified using the Agencourt AMPure XP magnetic beads in 25 µL of Low TE buffer. The exome pooled library was then clonally amplified by emulsion PCR following the Life Technologies SOLiD EZ bead E80 protocols (PN 4441486 Rev. D, 4443494 Rev. D, 4443496 Rev. D; Thermo Fisher Scientific). Emulsion PCR beads (300 µL) were 3' modified and deposited on three lanes of a FlowChip. The forward 75-bp reads and the Exact Call

Figure 1 The presence of additional germline mutations in a Li-Fraumeni family with a *TP53* germline mutation. *MSH4* mutations were detected in all six nervous system tumors analyzed, whereas *LATS1* mutations were observed in two schwannomas. Circles, females; squares, males; Numbers below symbols represent age at diagnosis. Tumor abbreviations: AA, anaplastic astrocytoma; ACC, adrenocortical carcinoma; BT, brain tumor (no histological verification); GBM, glioblastoma; WBC, white blood cells.

Chemistry sequencing was applied on a SOLiD 5500XL Genetic Analysis System (Applied Biosystems; Thermo Fisher Scientific).

Exome Data Analysis

Exome data analyses (mapping and variant calling) were performed using a combination of LifeScope Genomic Analysis software version 2.5.1 (Thermo Fisher Scientific) targeted resequencing and low frequency variant detection workflows with the default parameters: the color space reads generated by the SOLiD5500XL were mapped against the human genome reference version hg19, generating bam files that were then enriched for targets and good-quality reads, and single-nucleotide variants (SNVs) were called on enriched bam files in both targeted resequencing and low-frequency variant detection, and small insertions and deletions (indels) were called on enriched bam files in targeted resequencing workflow (Thermo Fisher Scientific). The variant call format output files were then annotated using the Annovar software (July 2013 version), including functional annotations as well as frequencies in known databases and in our custom SOLiD catalog of variants. Finally, the variant call format files were annotated using a “back-to-bam” script that added the number of reads found at each position, to each allele and on each strand.

To remove recurrent false-positive calls or common variants, we excluded from the annotated list of variants those found in >10% of our mutations list generated from our own SOLiD exome sequencing data from 65 different samples. Additional quality filtering includes exclusion of variants with: i) read depth below five, ii) numbers of different starting points below three, and iii) genomic segmental duplications (reflecting repetitive genomic regions) over 0.9 (this filter applied to SNVs only). Common annotated variants in both patients, F and G, with unknown frequency or a reported frequency of <1% in the 1000 Genomes database (<http://www.1000genomes.org>, data retrieved June 21, 2010) were then further filtered based on the following refGene annotations criteria: i) inclusion of SNVs that generate or abrogate exonic nonsense mutations, ii) inclusion of small indels that generate exonic frameshift and nonframeshift mutations, iii) inclusion of nonsynonymous SNVs with a predictive deleterious score in SIFT or PolyPhen-2 tools,^{8,9} and iv) inclusion of SNVs that disrupt the splicing process. Candidate genes were then ranked using DAVID functional annotations tools (December 18, 2009), ToppGene candidate gene prioritization tool (ToppGene Suite, <http://toppgene.cchmc.org/prioritization.jsp>, data retrieved May 11, 2009), or the UCSC database (UCSC Genome Bioinformatics, <http://genome.ucsc.edu>, data retrieved April 3, 2002). Genes involved in carcinogenesis, cell cycle, cell proliferation and apoptosis, cell division, cell growth, DNA repair, cell differentiation, microtubule cytoskeleton organization, or cerebral development were prioritized for further validation. Moreover, genes with a high expression level in the central

nervous system and/or that involved the pathway or complex known to play a role in the development of schwannomas [Salvador/Warts/Hippo (SWH) pathway and SMARCB1 SWI/SNF complex] were added to the list of candidate genes.

Validation of Genetic Alterations by Sanger Sequencing

We performed Sanger sequencing to validate results obtained by exome sequencing. We first analyzed DNA samples extracted from blood samples from patients F and G, as well as those from blood samples of four healthy individuals unrelated to this family. Genetic alterations found to be present in the blood DNA samples from patients F and G, but not in the four blood DNA samples from healthy individuals, were considered for validation.

We then assessed, by Sanger sequencing, genetic alterations in validated genes in six nervous system tumors (anaplastic astrocytoma and glioblastoma in patient I, glioblastoma in patient D, anaplastic astrocytoma in patient E, and schwannomas in patients F and G) for which samples were available. PCR products were subjected to direct sequencing on an ABI 3100 PRISM DNA sequencer (Applied Biosystems; Thermo Fisher Scientific) with the ABI PRISM BigDye Terminator Cycle Sequencing Kit (Applied Biosystems; Thermo Fisher Scientific), as previously reported.¹⁰

Analysis of Microsatellite Instability

Microsatellite instability was assessed using mononucleotide repeats CAT25, BAT25, and BAT26 as described by Findeisen et al.¹¹ Sense primer for BAT25 was labeled at the 5' end with HEX fluorescent dye, and sense primers for BAT26 and CAT25 were labeled with FAM fluorescent dye. DNA samples from colon carcinoma cell line LoVo carrying a *MSH2* homozygous mutation and prostate cancer cell line DU-145 carrying a *MLH1* homozygous mutation, which show microsatellite instability,¹² served as positive controls. DNA samples of two healthy individuals unrelated to this family were used as negative controls.

Ten-nanogram DNA samples from tumor tissues of patients E, F, and G, those from the blood of patients F and G, and positive- and negative-control DNA samples were subjected to multiplex PCR with each primer at a final concentration of 0.5 $\mu\text{mol/L}$, as previously described.¹¹ Fragment analysis was performed on an ABI3730XL genetic analyzer with 500 LIZ size standard (Applied Biosystems; Thermo Fisher Scientific) using Filter set E. Allelic sizes were visualized and analyzed using Peak Scanner software version 1.0 (Applied Biosystems; Thermo Fisher Scientific). Samples in which at least two of the three markers displayed a large shift of product size were considered as high-level microsatellite instability, whereas those with one positive marker showing abnormal size were interpreted as low-level microsatellite instability.¹¹

Methylation-Specific PCR for *LATS1* Promoter Methylation in Schwannomas

Promoter methylation of *LATS1* was determined by methylation-specific PCR.¹³ DNA samples from two schwannomas from patients F and G, positive controls (Universal Methylated DNA; Intergen, Edinburgh, UK), and negative controls (DNA extracted from normal blood samples) were treated with bisulfite using the EZ DNA Methylation Kit (Zymo Research, Irvine, CA), as described previously.¹⁴ Methylation-specific PCR was performed in a C1000 Touch Thermal Cycler (Bio-Rad Laboratories, Hercules, CA) in a total volume of 10 μ L, with initial denaturing at 95°C for 5 minutes, followed by 40 cycles consisting of denaturing at 95°C for 30 seconds, annealing at 54°C (for methylated reaction) or 58°C (for unmethylated reaction) for 30 seconds, and extension at 72°C for 45 seconds. A final extension step at 72°C for 5 minutes was added. Primer sequences are 5'-TTTGTATTATACGATTCGTTTCGC-3' (forward) and 5'-CCCTTCTAAAAACGTAACCTCCGTA-3' (reverse) for the methylated reaction (PCR product, 144 bp) and 5'-TTTTTGTATTATATGATTTGTTTGTGT-3' (forward) and 5'-ATTCCCTTCTAAAAACATAACTCCAT-3' (reverse) for the unmethylated reaction (149 bp). PCR products were electrophoresed on 2% agarose gels and were visualized with GelRed Nucleic Acid Gel Stain (Bio-Rad Laboratories, Hayward, CA). Positive and negative controls were included for each reaction.

Screening for *LATS1* Mutations in Sporadic Schwannomas

We screened for *LATS1* mutations using single-strand conformation polymorphism followed by Sanger sequencing in 65 sporadic schwannomas, 12 hybrid neurofibroma/schwannoma tumors, and 4 cases of schwannomatosis. Thirty sets of primers were designed to cover exons 2 to 8 of the *LATS1* gene (Table 1). Samples showing mobility shifts were subjected to Sanger sequencing using the same primers used for single-strand conformation polymorphism. To avoid false-negative and false-positive results, positive controls with known mutations and water served as positive and negative controls, respectively, and were included in each analysis.

Results

Exome Sequencing and Selection of Candidate Genes

We generated 6.7 Gb and 5.6 Gb of DNA data from peripheral blood samples of patients F and G, respectively, with an average of 96% of the reads mapping to the reference genome. The percentage of the numbers of reads within targets was 71% and 73%, and the average coverage of reads within target was 77.8 $\times$ and 66.5 $\times$ for patients F and G, respectively. On the basis of our filtering criteria, we obtained 128 SNVs (85 identified by both LifeScope workflows and

43 identified with the targeted sequencing workflow only) and nine small indels that were carried by both patients F and G. Of the 128 SNVs, 115 (90%) were missense substitutions, 7 (5.5%) were nonsense mutations, and 6 (4.7%) were SNVs located at splicing sites. Of the nine small indels, three were frameshift deletions, three were frameshift insertions, one was a deletion leading to a stop codon, and two were small indels, leading to the disruption of splicing sites.

We prioritized candidate genes according to their functions using DAVID functional annotations tools, ToppGene candidate gene prioritization tool, and UCSC database. Genes involved in carcinogenesis, cell cycle, cell proliferation, apoptosis, cell division, cell growth, DNA repair, cell differentiation, microtubule cytoskeleton organization, and those expressed at a high level in the central nervous system were considered to be high priority. Eleven genes (*MSH4*, *ATP2B2*, *HAUS1*, *ESPL1*, *LATS1*, *TP53BP2*, *GRP*, *UBA6*, *RBPJL*, *CAPNS2*, and *EPB41L1*) were chosen for validation by Sanger sequencing.

Validation of Genetic Alterations by Sanger Sequencing

Sanger sequencing confirmed genetic alterations in six genes (*MSH4*, *LATS1*, *HAUS1*, *CAPNS2*, *RBPJL*, and *ATP2B2*) in blood samples from patients F and G, but not in normal DNA samples from four unrelated healthy individuals. These were a missense mutation in *MSH4* at 1p31.1 (c.2480T>A; p.I827N) (Figure 2), a missense mutation in *LATS1* at 6q25.1 (c.286C>T; p.R96W) (Figure 2), and a missense mutation in *ATP2B2* at 3p25.3 (c.1414C>T; p.R472C). Analyses using the SIFT or PolyPhen-2 tools^{8,9} suggested that these missense mutations are biologically deleterious, ie, probably damaging protein structures and functions. Other alterations were a frameshift mutation in *HAUS1* at 18q21.1 (c.327-328 insA, p.D109fs; insertion A), leading to a stop codon at codon 114, a nonsense mutation in *CAPNS2* at 16q12.2 (c.238C>T; p.R80X), and a nonsense mutation in *RBPJL* at 20q13.12 (c.C>239A; p.S80X). All these mutations were heterozygous, with a wild-type sequence in the remaining allele. For *MSH4* and *LATS1* genes, we further assessed normal DNA samples from an additional 10 healthy individuals and confirmed that all were negative.

We then screened for these alterations in nervous system tumors in the studied family. The *MSH4* mutation (c.2480T>A; p.I827N) was present in all six nervous system tumors (two anaplastic astrocytomas, two glioblastomas, and two schwannomas) analyzed. The *CAPNS2* mutation (c.238C>T; p.R80X) was observed in an anaplastic astrocytoma and in two schwannomas. Mutations in the *LATS1*, *HAUS1*, *RBPJL*, and *ATP2B2* genes were detected only in two schwannomas.

Analysis of Microsatellite Instability

Analysis using T₂₅ repeat in the 3' untranslated region of the *CASP2* gene (CAT25) and the standard NCI/ICH-HNPCC

Table 1 Primer Sequences of the *LATS1* Gene

	Primer sequences	Annealing temperature	PCR product
Exon 2-1	F: 5'-ATAAAGAAGTCCTTCGTGTGG-3' R: 5'-AGCAGCATCAGATGGTTTAGA-3'	55°C	188 bp
Exon 2-2	F: 5'-AGCCGGCAAATGTTACAAGA-3' R: 5'-TTTGCAAATGGAAGCAGAGA-3'	58°C	191 bp
Exon 2-3	F: 5'-ATCATAAAGCCTTGCAGGAA-3' R: 5'-AAAGTGTTATGTAGCATAAGG-3'	55°C	187 bp
Exon 3-1	F: 5'-TTCCCTTGTATTATTGTAAG-3' R: 5'-TCTGCTCTCGTCGAGGATCT-3'	55°C	167 bp
Exon 3-2	F: 5'-CAGAAGTATAGAAGCAGCAATTG-3' R: 5'-ACACAGTAAAAGAAGAGATGAGA-3'	55°C	159 bp
Exon 4-1	F: 5'-CAAAAATTAGAGGTTAAAAACATG-3' R: 5'-CTGTGAGTTGGGACTCTCAGAA-3'	55°C	187 bp
Exon 4-2	F: 5'-CTTAGTTCCTCAGAGGCATGG-3' R: 5'-TCCTTACTTGAGGTGGTGGTG-3'	61°C	179 bp
Exon 4-3	F: 5'-TCAAGCTCACCTAGCAACG-3' R: 5'-CGGGAGATTACGTATTCCATGT-3'	55°C	190 bp
Exon 4-4	F: 5'-CCTTCATGGGAACCAAACCTC-3' R: 5'-TTGTCTGCCAACAGGAACAG-3'	55°C	189 bp
Exon 4-5	F: 5'-CAACACTTCCCCCATGAATC-3' R: 5'-GCAGGGACAACATTTTGGTG-3'	55°C	180 bp
Exon 4-6	F: 5'-AACTTTCCATCAGGGAGACCT-3' R: 5'-ATGACGAAGGAGCAGCAGAT-3'	55°C	184 bp
Exon 4-7	F: 5'-CAGCAGCTAATGGACAAAGC-3' R: 5'-GGCTGGAGCAGAAGATGACT-3'	55°C	188 bp
Exon 4-8	F: 5'-CATTAGTGACCTGGACTGCA-3' R: 5'-CTGTTGTAGCAGAAGGCTGAGA-3'	55°C	188 bp
Exon 4-9	F: 5'-GGTCAAATTCCTTTAATAACCCA-3' R: 5'-GGCTGTGGTATCCAAGAAGG-3'	55°C	187 bp
Exon 4-10	F: 5'-TGCGTGTATTAAACCAGAGC-3' R: 5'-ATGTTTTGGGTAGGGTGGTG-3'	55°C	185 bp
Exon 4-11	F: 5'-CCTGTTGCTGAAGCTCCAAA-3' R: 5'-TCTTTATCCCCACTATCAACA-3'	55°C	185 bp
Exon 4-12	F: 5'-CAAGCTTGCCCAAGGAAGA-3' R: 5'-TCTACATGTTGCTCCATAAAGA-3'	55°C	190 bp
Exon 4-13	F: 5'-CGAAGGGAATCTCGTATTCAA-3' R: 5'-AACACCAAGCAAACAGATGAT-3'	55°C	190 bp
Exon 5-1	F: 5'-CAATATTGAAGAAGATAATTCT-3' R: 5'-CAGACTTCACCAAATGCTCCT-3'	55°C	197 bp
Exon 5-2	F: 5'-AGGGCTAAAAATGGACAAGTCT-3' R: 5'-TGTCAGCTTCAGCCAGGATA-3'	55°C	196 bp
Exon 5-3	F: 5'-GAAAGATGTTCTTCTTCGAAATC-3' R: 5'-TTCTGGAAAGATGCCCATTCT-3'	55°C	193 bp
Exon 5-4	F: 5'-GACTACATTCTGGGGGTGA-3' R: 5'-TGACCATCACGATCAATCAAA-3'	55°C	176 bp
Exon 5-5	F: 5'-ATGGGTTTTATTCATAGAGA-3' R: 5'-CATCAATAAATTACATTATTATGAAC-3'	55°C	185 bp
Exon 6-1	F: 5'-TCAGGTTTACTGTTGTCATTGTTG-3' R: 5'-TGTGCTAGACATCGCTGGTG-3'	55°C	184 bp
Exon 6-2	F: 5'-GCTGTCGATGTGGAGACAGA-3' R: 5'-AGTGTCTGTTTTGGCTGGTCT-3'	55°C	185 bp
Exon 7-1	F: 5'-GTATTCTGAGCATTTTAAAGTGC-3' R: 5'-GCAACTTTGTGAGAATTCTTTTGA-3'	55°C	198 bp
Exon 8-1	F: 5'-CTAATTTATCTCTTCCCCTA-3' R: 5'-TGGATGAGCTTTTATTTTCATCA-3'	55°C	182 bp
Exon 8-2	F: 5'-CGAAGATCGCTTAGGCAAGA-3' R: 5'-CTTCCTCGTTATCATCACTCCA-3'	55°C	188 bp

(table continues)

Table 1 (continued)

	Primer sequences	Annealing temperature	PCR product
Exon 8-3	F: 5'-TTTGTATCCTGTTGATCCTGA-3' R: 5'-TCATATTCAATAGGCTTCGGA-3'	55°C	186 bp
Exon 8-4	F: 5'-TTTTTGATGACAATGGCTACC-3' R: 5'-GCATTTTCAGGCCCTTTTACA-3'	55°C	194 bp

F, forward; R, reverse.

marker panel (BAT25 and BAT26) revealed no microsatellite instability in any of the samples from patients with *MSH4* germline mutations (an anaplastic astrocytoma from patient E; blood samples and schwannoma tissues from patients F and G).

Two negative-control DNA samples from two unrelated healthy individuals showed normal ranges of the PCR product sizes in three markers (approximately 146 to 148 bp for CAT25, approximately 120 to 122 bp for BAT25, and approximately 116 to 118 bp for BAT26), as previously reported,¹¹ whereas colon cancer cells carrying a *MSH2* homozygous mutation and prostate cancer cells carrying a *MLH1* homozygous mutation showed PCR product sizes 4 approximately 10 bp shorter than the normal range in all three markers, indicating high-level microsatellite instability.

LATS1 Promoter Methylation in Schwannomas with a *LATS1* Germline Mutation

The sequencing pattern showed *LATS1* germline mutation in one allele with a remaining wild-type allele in two schwannomas from patients F and G. Because the *LATS1* gene has CpG islands, and promoter methylation has frequently been reported in a variety of neoplasms, including gliomas,¹⁵ we assessed *LATS1* promoter methylation in 2 schwannomas and blood samples from patients F and G. Methylation-specific PCR revealed *LATS1* promoter methylation in schwannoma tissues, but not in blood samples, in both patients.

LATS1 Mutations in Sporadic Schwannomas

Because *LATS1* is a downstream mediator of the *NF2*, which is frequently mutated in schwannomas, we screened the entire coding sequence of the *LATS1* gene in sporadic schwannomas. Screening for *LATS1* mutations by Sanger

Figure 2 *MSH4* and *LATS1* germline mutation in schwannoma patients in a family. *LATS1* mutation (1-bp deletion) in a sporadic schwannoma.

sequencing revealed a single base deletion at codon 827 in exon 5 (c.2480delG; p.*R827Kfs*8), leading to a stop codon at codon 835 in one of 36 (3%) spinal schwannomas analyzed (Figure 2). No mutations were found in any of 29 peripheral schwannomas, 4 cases of schwannomatosis, or 12 hybrid tumors with neurofibroma/schwannoma.

Common polymorphisms in the *LATS1* gene were observed at codon 203 (1 case), codon 482 (10 cases), codon 857 (1 case), and codon 1014 (8 cases). Analyses using the Exome Variant Server database (NHLBI GO Exome Sequencing Project, <http://evs.gs.washington.edu/EVS>, data retrieved May 21, 2012) showed no significant differences in frequencies of any of these single nucleotide polymorphisms between schwannomas analyzed in the present study and those in healthy populations.

Discussion

The occurrence of multiple germline mutations as identified in this family is quite unique. We previously reported the clustering of brain tumors caused by a *TP53* germline deletion of codon 236.^{5,6} All family members with a nervous system tumor (four gliomas and two schwannomas) also carried a *MSH4* germline mutation (c.2480T>A; p.I827N). To our knowledge, this is the first report of *MSH4* germline mutations. The *MSH4* I827N mutation is located in the highly conserved ATPase domain and is considered to be biologically deleterious, probably by damaging protein structures and functions, according to SIFT and PolyPhen-2 tools.^{8,9}

MSH4 belongs to a member of the DNA mismatch repair mutS family.^{16,17} Mutations of several mutS family genes (*MSH2* and *MSH6*) have been reported to cause Turcot syndrome type 1 (nonpolyposis colorectal carcinoma and glioblastoma).^{1,18} Cancer cells with *MSH2* or *MSH6* mutations show DNA mismatch repair deficiency leading to microsatellite instability.^{19,20} By contrast, there has been no clear evidence of the involvement of *MSH4* in DNA mismatch repair.^{21,22} *MSH4* forms a unique and exclusive heterocomplex with *MSH5*, but not with other members of the DNA mismatch repair mutS family.²² *MSH4* and *MSH5* are considered to play important roles in meiotic and mitotic DNA double-strand break repair and DNA damage responses.¹⁶ Chu et al²³ showed evidence that *MSH4* is involved in the process of DNA damage response and that it promotes genomic stability by restricting the use of

error-prone nonhomologous end joining following double-strand breaks. Several studies suggested that MSH4 and MSH5 play distinct roles through interacting with different binding partners.²² We assessed microsatellite instability in three nervous system tumors (two schwannomas and one anaplastic astrocytoma) from patients with *MSH4* germline mutations, and showed lack of microsatellite instability, confirming the view that MSH4 is not involved in DNA mismatch repair.

Somatic *MSH4* mutations have been reported in tumors of the large intestine (6 of 18; 33%)²⁴ and skin (2 of 8; 25%),²⁵ but are rare in ovarian cancer (2 of 59; 3%)²⁶ and lung cancer (3 of 146; 2%).²⁷ No *MSH4* mutations have been reported in 564 central nervous system tumors (COSMIC, <http://www.sanger.ac.uk/genetics/CGP/cosmic>, data retrieved October 15, 2010).^{28,29} Similarly, The Cancer Genome Atlas Data Portal data show an absence of *MSH4* mutations in 122 glioblastomas analyzed, whereas *MSH2* and *MSH6* mutations were found in 2% and 3% of cases, respectively (The Cancer Genome Atlas Data Portal, <https://tcga-data.nci.nih.gov/tcga>, data retrieved September 4, 2008). This indicates that *MSH4* mutations alone may not be sufficient to initiate the development of nervous system tumors.

This family is extraordinary because two siblings lacking a *TP53* germline mutation developed peripheral schwannomas, benign nerve sheath tumors composed of differentiated neoplastic Schwann cells. These patients also had a *MSH4* germline mutation, but the contribution of this genetic alteration to the development of schwannomas is unclear. Bilateral vestibular schwannomas are diagnostic of the neurofibromatosis type 2 syndrome, an autosomal dominant disorder characterized by an *NF2* germline mutation.^{1,30} Somatic *NF2* mutations and loss of 22q12.2 at the *NF2* locus are frequent in sporadic schwannomas (approximately 60%).^{1,31,32} Other, less frequent genetic alterations in sporadic schwannomas include *SMARCB1/INI1* (up to 10%),^{33,34} loss at 1p (approximately 10%),³⁵ and gain at 9q (approximately 10%).³⁶ However, there are no reports on *MSH4* mutations in sporadic schwannomas.

Exome sequencing of peripheral blood DNA revealed *LATS1* germline mutations in both schwannoma patients, but not in any other family members analyzed. *LATS1* encodes a Ser/Thr kinase, which is a downstream molecule of NF2 in the SWH pathway, and plays a role in modulating organ size by regulating cell proliferation and apoptosis, and a role in the regulation of genetic and protein stability.^{37,38} LATS signaling intersects with well-established tumor suppressive and oncogenic pathways, including the p53, Ras, or Akt networks.³⁹ In addition to the SWH pathway, LATS also acts independently of this pathway, possessing multiple functions, including regulation of cell proliferation, cell death, and cell migration; transcriptional regulation; and maintenance of genetic stability.³⁹ Mice deficient in *LATS1* develop soft tissue sarcomas, ovarian tumors, and pituitary dysfunction.⁴⁰

To assess whether *LATS1* alterations play a role in the development of sporadic schwannomas, we screened the

entire coding region of the *LATS1* gene in 65 sporadic spinal and peripheral schwannomas. We only found a single base deletion at codon 827 in exon 5 (AGA → AA), leading to a stop codon at codon 835 in 1 of 36 (3%) spinal schwannomas. This suggests that mutational loss of function of *LATS1* may play a role in some inherited schwannomas, but only exceptionally in sporadic schwannomas.

No *LATS1* mutations have been reported in 548 central nervous system tumors analyzed (COSMIC).^{28,29} However, Ji et al⁴¹ showed that mRNA and LATS1 protein expression is significantly down-regulated in gliomas compared with normal brain tissue, and that decreased LATS1 expression correlated with the progression and prognosis of gliomas.

In two schwannomas carrying a *LATS1* germline mutation, sequencing patterns suggest one mutated allele, with a wild-type sequence in a remaining allele. Because LATS1 expression is lost or reduced largely because of promoter methylation in a variety of neoplasms, including gliomas,¹⁵ we assessed *LATS1* promoter methylation in 2 schwannomas in patients F and G (Figure 1). Both schwannomas, but not the blood samples, showed *LATS1* promoter methylation. This suggests that both alleles of *LATS1* were inactivated in schwannomas, one by a germline mutation and another by promoter methylation.

The presence of three different germline mutations in one family raises a question about the sequence in which these genetic changes developed. The *MSH4* mutation was present in all patients with a nervous system tumor. The *TP53* mutation in this family was unusual because an entire codon was deleted. We favor the hypothesis that the inactivation of MSH4 in germ cells may have played a role in the acquisition of additional *TP53* and *LATS1* germline mutations.

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