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# Comparison of Benign and Malignant Pilomatricomas Using Whole-exome Sequencing

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**Abstract.** *Background: Malignant pilomatricoma (MP) is a rare cancer of the hair matrix with only a few cases reported in literature. Given the rarity of this cancer and the lack of relevant genetic data, very little is known about the nature of the molecular pathophysiology except the involvement of the Catenin Beta 1 (CTNNB1)/Wnt/β-catenin signaling pathway in some cases. Materials and Methods: We describe the whole-exome genomic profiling of four samples from two patients: 1) an MP from patient I, 2) a coexisting benign pilomatricoma (BP) from patient I, 3) a BP from an age and location-matched control patient II, and 4) normal skin tissue from patient II. Results: We detected a pathogenic somatic missense mutation in fibroblast growth factor receptor 4 (FGFR4) (c.1162G>A, p. Gly388Arg) in MP and coexisting BP in patient I, whereas the control BP harbored the classical CTNNB1 mutant. Conclusion: This study, the first comparative analysis of benign and MP through whole-exome analysis, identified a novel oncogenic mutation in FGFR4.*

Malignant pilomatricomas (MPs) are extremely rare adnexal cancers originating from hair matrix cells. Fewer than 150 cases of MPs have been reported in literature worldwide and the vast majority as case reports (1-4). MP has significant recurrence and metastatic potential, but due to its slow growth and rarity, it is often clinically misdiagnosed as a sebaceous cyst or other common skin tumor, potentially delaying tumor resection (4). MP can arise *de novo* as a solitary lesion or through the transformation of a benign pilomatricoma (BP); however, no available immunohistochemical or molecular methods are capable of distinguishing BP from MP. Consequently, the diagnosis of malignancy must be based on histological examination (1). To date, the CTNNB1/Wnt/β-catenin signaling pathway, identified through selective molecular analysis, is the only tumorigenic factor shown to drive pilomatricoma (3, 5).

Here, we report the first case of MP subjected to whole-exome and mutational signature analysis. To obtain insight into the molecular mechanisms that might be responsible for malignant transformation, we compared the detailed molecular and immunologic characteristics among four samples from two patients: 1) malignant pilomatricoma (patient I), 2) coexisting benign pilomatricoma (patient I), 3) benign pilomatricoma (patient II), and 4) normal skin tissue (patient II). All cases were subjected to whole-exome sequencing (WES), mutational signature (MS) analysis, Sanger sequencing, and immunohistochemical studies.

## Materials and Methods

*Patients and tissue samples.* The MP sample, from patient I, was identified by retrospectively searching the pathology database at Chungnam National University Hospital, Daejeon, South Korea, with approval from the hospital's Institutional Review Board (2020-07-063). An age- and site-matched benign pilomatricoma (patient II) was selected as a control for this study, also from the case files of Chungnam National University. All available slides were reviewed by two licensed pathologists (Bae and Yeo). Clinical features and follow-up were obtained by review of the patients' electronic medical records. This study (2020-07-063) was reviewed and approved by the Institutional Review Board at the Chungnam National University Hospital, Daejeon, Republic of Korea.

*Whole-exome sequencing and data analysis.* Three benign and malignant pilomatricomas and one control normal skin sample underwent WES, performed as an outsourced service by Macrogen (Seoul, Republic of Korea). DNA was extracted from formalin-fixed, paraffin-embedded (FFPE) tissue sections. High-quality DNA (200 ng) was subjected to library preparation and exome capture using the SureSelect Target Enrichment System for Illumina Paired-End Multiplexed Sequencing Library (Illumina, San Diego, CA, USA). Captured material was indexed and sequenced on the Illumina HiSeq2500 platform, yielding an average sequence coverage of >40×. All procedures were performed according to the manufacturer's instructions as reported in the literature (6, 7). The



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*Key Words:* CTNNB1, FGFR4, malignant, mutation, pilomatricoma, whole-exome sequencing.

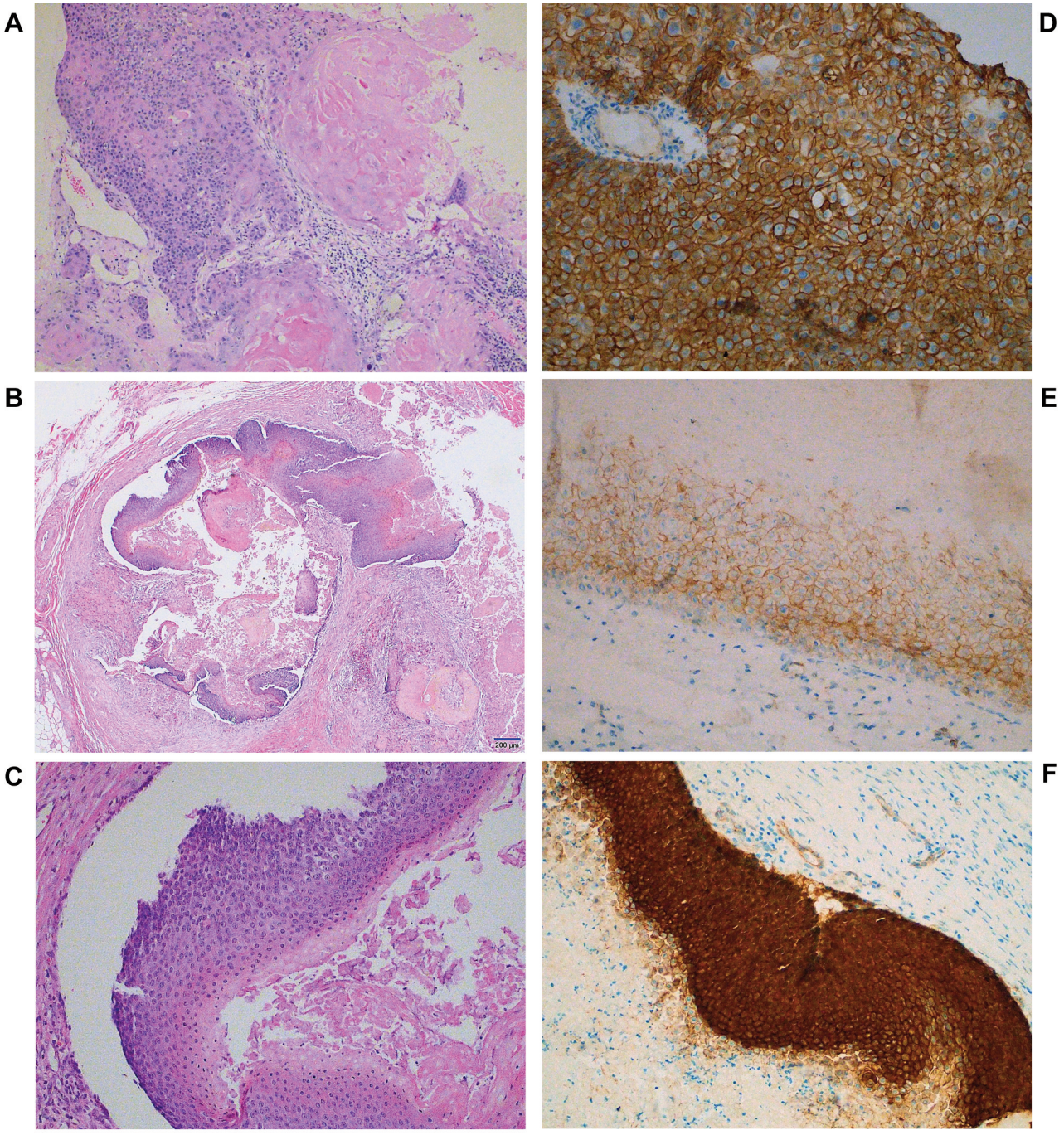


Figure 1. *Continued*

resultant FASTQ files were aligned to the National Center for Biotechnology Information (NCBI) build 37.2 of the human reference genome (hg19) with default paired-end parameters. Quality metrics were calculated using the MuTeck2 software. (v4.1.0.0, CA, USA), and sequence data were arranged to mark duplicate reads and recalibrate quality scores, and were realigned around known polymorphisms using the Genome Analysis Toolkit (GATK v3.8, CA, USA) (8). The extracted data were further interpreted to standardize pathogenic clinical significance according to ClinVar-indexed variants (NCBI, USA) (9). A variant was considered positive if pathogenic or likely pathogenic variants corresponded to the phenotype and the mode of inheritance (10, 11). *Sanger sequencing*. The MP underwent direct sequencing as an outsourced service by Macrogen (Seoul, Republic of Korea). DNA

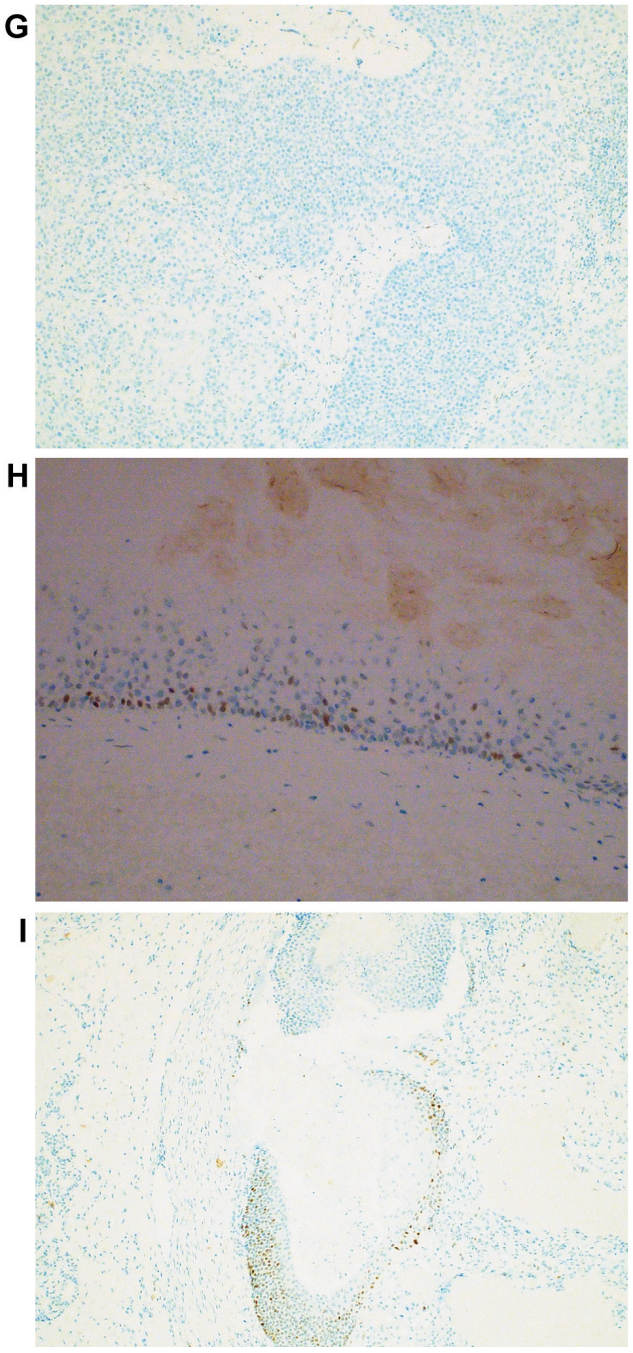


Figure 1. *Pathologic and Immunohistochemical (IHC) features of benign and malignant pilomatricomas. (A) Malignant pilomatricoma (MP) presents basaloid cell nests with stromal invasion and immature keratinization [100×, Hematoxylin-eosin stain (HE)]. BP from patient I (B) and control patient II (C) reveal keratinizing basaloid cell nests without cytological atypia. (D-F) Expression patterns of β-catenin IHC differ between two patients; membranous staining in patient I [MP (D) BP (E)] vs. strong nuclear and cytoplasmic expression in BP from control patient II (F, 200×). p53 expression patterns differed between benign and malignant tumors (G-I: negative (null) staining in MP (G) vs. wild-type expression in both BPs (H from patient I, I from control patient II).*

was extracted from an FFPE block from the MP and quantified by QIAxpert (QIAGEN, Hilden, Germany) analysis, yielding a concentration of 28.4 ng/μl. A fragment of the human *FGFR4* exon 9 was amplified with PCR products that were cloned into plasmids and sequenced using the BigDye(R) Terminator v3.1 Cycle Sequencing Kit (CE Sequencing Instruments, San Jose, CA, USA). Sequence identification and variant analysis were performed using Variant Reporter 2.1 (Applied Biosystems, CA, USA) and DNASTAR Lasergene SeqMan 7.0 (WI, USA). For all analyses, data obtained with the forward and reverse primers were combined and aligned to the consensus sequence obtained from the GenBank database (NCBI Reference Sequence: NG_012067.1).

*Mutational signature analysis*. To obtain additional clues about the causative origins of cancer, extracted somatic point mutations were mapped to the 96 possible trinucleotide contexts for signature analysis (12). To reconstruct the mutation profile of each sample, the data were then analyzed with DeconstructSigs 1.9, a multiple linear regression-based algorithm, using a linear combination predefined by the Signatures of Mutational Processes in Human Cancer database found at http://cancer.sanger.ac.uk/cosmic/signatures (12).

*Immunohistochemical studies*. FFPE tissue blocks of four samples from two patients (patient I: MP and coexisting BP; patient II: BP and normal skin tissue) were sectioned at 4 μm thickness. Sections were deparaffinized in xylene and rehydrated through a graded alcohol series. Immunohistochemical staining (IHC) was performed using an automatic immunostaining instrument (Ventana Benchmark XT; Ventana Medical Systems, Tucson, AZ, USA). Commercially available antibodies were used including β-catenin (Invitrogen, Carlsbad, California, USA; 1:500 dilution) and p53 (DAKO, Glostrup, Denmark; 1:300 dilution).

## Results

*Clinicopathologic characteristics*

*Patient history*. A 43-year-old woman with no significant past medical history presented with a palpable nodule under the superior scalp that had been present for a few years. Upon a radiologic CT scan, the nodules exhibited a well-confined subcutaneous cystic lesion without peripheral soft tissue invasion. The radiologist suggested BP and epidermal cyst as differential diagnoses. Excisional biopsy of the lesion revealed a well-circumscribed, dome-shaped, whitish cystic tumor containing necrotic inner contents, measuring 2.1 cm in maximum diameter, mainly located in the dermis and subcutaneous tissue of the excised skin. The diagnosis of MP was supported by the presence of focal pleomorphic atypical cell nests with multiple abnormal mitosis (Figure 1A) admixed with the BP (Figure 1B, BP1), as determined by microscopy. The findings suggested malignant transformation within the coexisting BP. The pathology of age- and site-matched benign control pilomatricoma (BP2) is shown in Figure 1C.

*Immunohistochemical findings*. The IHC expression patterns of the β-catenin from the two patients are distinct: membranous expression in MP (Figure 1D) and coexisting BP1 (Figure 1E) from patient I *vs*. strong nuclear and cytoplasmic β-catenin

expression in BP2 from the control patient (Figure 1F). By contrast, the patterns of p53 expression differ between the benign and malignant tumors: completely negative expression in MP (Figure 1G), indicative of functional loss due to *TP53* mutation, *vs*. wild-type p53 expression in both BPs from the two patients (Figure 1H, I). In addition, we performed IHC studies to screen for abnormalities in DNA mismatch repair proteins, including MLH1, MSH2, PMS2, and MSH6; in all cases, the results revealed no microsatellite instability (data not shown).

*Molecular analysis*

*Somatic variant profiling and mutational classification*. Three pilomatricomas and normal control skin tissue were sequenced and analyzed by WES. The average numbers of reads for tumor and normal samples were 256,668,105 and 114,853,678, respectively, generating an average coverage of 96.1× and 99× across 99% of the captured sequence. The average number of somatic missense mutations identified in exomes ranged from 11.439 to 11.623 (Figure 2A). Overall, the extracted data revealed that the types and amounts of somatic variants were similar between the two benign and one malignant pilomatricomas (Figure 2A). However, after filtering by mutational classification, only the *FGFR4* (c.1162G>A, p. Gly388Arg, NM 213647.3) missense mutation remained as a deleterious pathogenic mutation in both MP and the coexisting BP1 (Figure 2B). In contrast to previous studies, *CTNNB1* (c.98C>T, p. Ser33Phe, NM001904.4) was identified as a pathogenic mutation only in the control BP2 (patient II) (Figure 2B). We further examined the extracted data, focusing on somatic variants that have been suggested by previous studies to be potentially targetable genetic mutants in hair matrix and adnexal cancers including *TP53, HRAS, RB1, ATM, ARID1A, APC PTEN, EGFR, CDKN2A, CDKN2B, CDKN1A, ALPK1, DNMT3A, PIK3CA, CYLD, NOTCH1, NOTCH2, ZNF750, RREB1, KMT2D, KRAS, PTCH1, TCF7L1, MLH1, MSH2, MSH6, PMS2, POLE,* and *FAT3*. No significant pathogenic genomic alterations were identified among these genes by mutational classification (14-21). An additional splice-donor and intron variant in *TP53* (c.375+1G>A, NM 000546.5) was only present in the MP (Figure 2B).

*Sanger sequencing*. Exon 9 of *FGFR4* was additionally analyzed by Sanger (direct) sequencing from the remaining tumor tissue from MP and BP1. *FGFR4* mutation was confirmed at codon 388 (Gly388Arg) in both the forward and reverse sequences as a heterozygous single-nucleotide polymorphism (Figure 2C, red arrow).

*Mutational signature analysis*. Computational analysis of the MS allowed us to attribute mutations to environmental exposure and mutagens (22-24). The MP and coexisting BP1 shared most MS patterns, including single base substitution (SBS) 1, SBS12, SBS16, SBS32, and SBS39, whereas the control patient's BP had a different MS configuration, except for SBS1 (Figure 3A). The patterns and composition from the MP and coexisting BP1 were similar but had no predominant MS type, whereas the control BP2 had different MS patterns with predominance of SBS1 (>60%). The 96 mutational classes of all SNVs within the three tumors shared the main mutational processes: C>T changes represented SBS1, whereas minor T>C changes were identified as SBS12, 16, and 32 in tumors, except for the control tumor BP2 (Figure 3B).

## Discussion

Malignant pilomatricoma (MP), a very rare adnexal cancer with a high rate of local recurrence but uncommon distant metastasis, has not been extensively characterized at the molecular level. To date, *CTNNB1*, the gene encoding β-catenin, is the only driver mutation identified in both benign and MPs (3, 13). Magdalena *et al*. as well as Katharina *et al*. have reported a few pediatric cases of multiple pilomatricoma with *CTNNB1* mutations, associated with germline mutations in mismatch repair genes (14, 15). The reported frequency of the *CTNNB1* mutation in pilomatrical tumor varies from 30% to 100% (16, 17), raising the question of whether actionable mutations in pilomatrical tumors occur in genes other than *CTNNB1*.

In this study, we identified a novel *FGFR4* c.1162G>A missense mutation in MP and its coexisting BP through WES. *FGFR4* is normally highly expressed in embryonic tissues and is involved in embryonic development, angiogenesis, and tissue differentiation, whereas in adult tissues its expression is limited (18). *FGFR4* mutations activate oncogenic signaling pathways and have been observed in breast, colorectal, lung, and melanocytic skin cancers (29-31). The c.1162G>A mutation, which is predominant in these contexts, changes the encoded amino acid residue (codon 388 in exon 9, located in the transmembrane domain) from glycine to arginine, exposing a membrane-proximal signal transducer and activator of transcription 3 (*STAT3*) binding site that activates the downstream *STAT3* signaling cascade (19, 20).

In several cancers, Arg388 allele carriers have significantly poorer prognoses than homozygous Gly388 allele carriers (20). *FGFR4* Gly388Arg mutants are present in 55% of skin melanoma patients and 38% of patient-derived melanoma cell lines, and are correlated with poor tumor thickness and invasiveness in these cancers (21-23). The frequency of *FGFR4* mutation has not been evaluated in non-melanocytic skin cancer; hence, further studies are needed to assess the prognostic risks associated with *FGFR4* mutations in skin adnexal tumors, including pilomatrical ones (2).

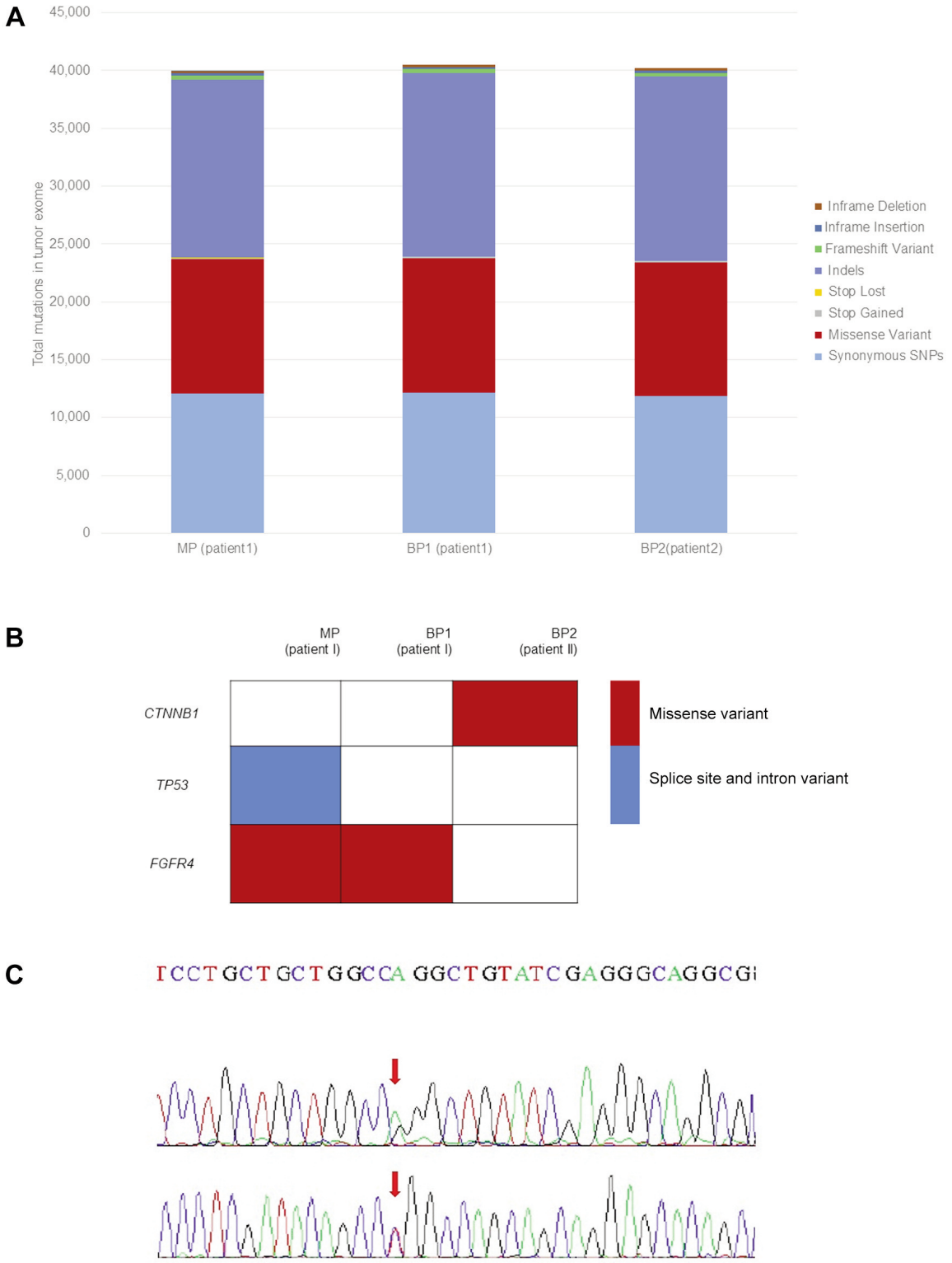


Figure 2. *Molecular profiling of pilomatricomas. (A) Overall amounts of somatic mutation and variant types were similar among all tumors. (B) After filtering by mutational classification, the pathogenic variants differed: FGFR4 in malignant pilomatricoma (MP) vs. benign pilomatricoma (BP1) from patient I, and CTNNB1 in control BP2. The TP53 splicing donor and intron variant were identified only in MP. (C) Chromatogram of exon 9 from FGFR4 gene obtained by Sanger sequencing with forward (upper) and reverse (lower) primer. The arrow indicates a clear heterozygous double peak (red arrow) at c.1198, generating the G388A missense mutation.*

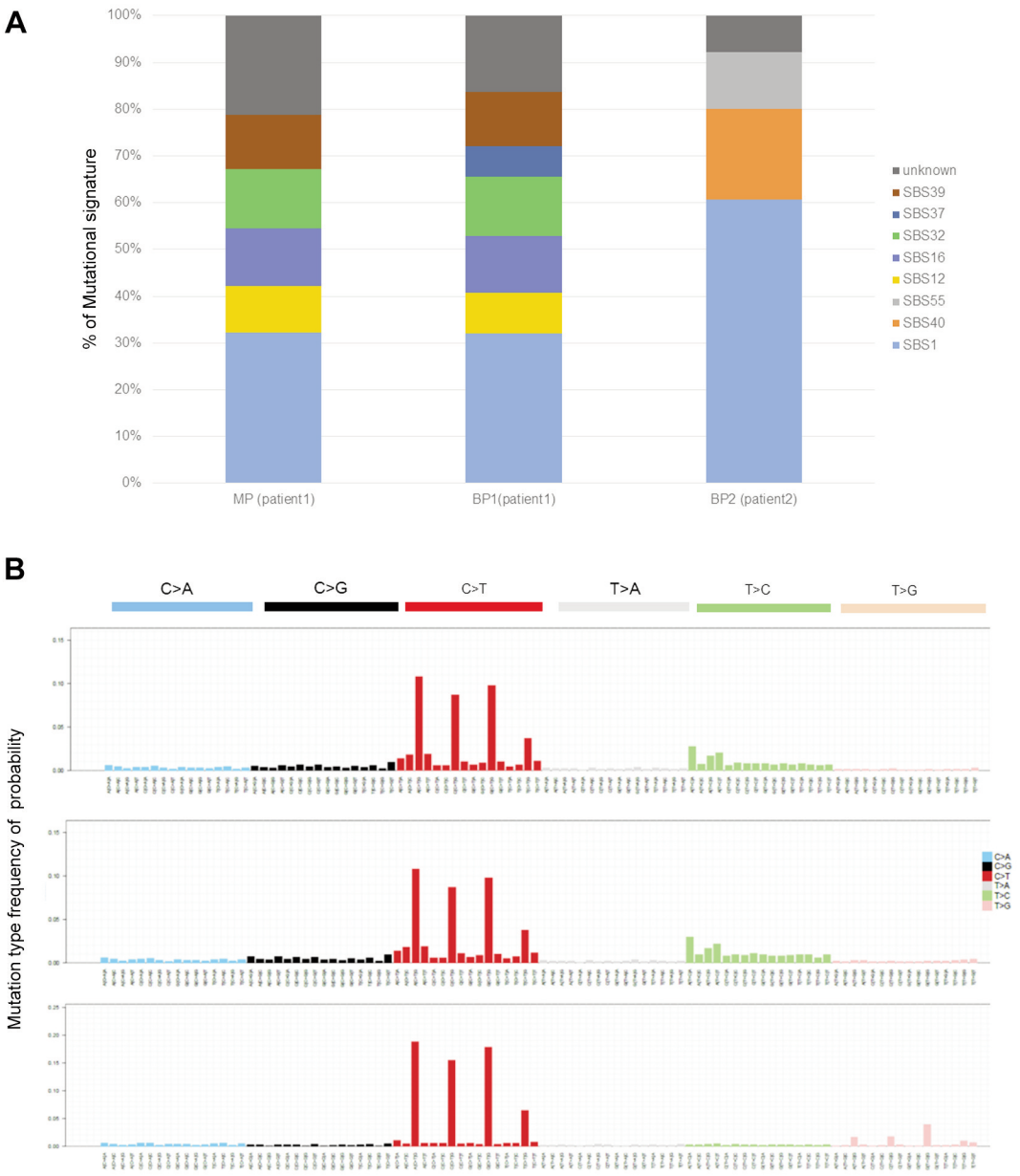


Figure 3. *Mutational spectra and signature analysis. (A) Bars show various mutational signatures (MS) extracted in pilomatrical tumors. Patterns and composition from MP and coexisting BP1 are similar but exhibit no predominant MS type, whereas control BP2 exhibit different MS patterns with predominant single base substitution (SBS) 1 (>60%). (B) The 96 mutational classes of all SNVs; no predominant sequence context was identified.*

The molecular pathogenesis of skin cancer remains under investigation, but UV radiation-induced damage is one of the most frequent oncogenic drivers causing impairment of immune suppression in skin cancers, which have the highest mutational burden (2, 24). Because pilomatrical tumors frequently occur in sun-exposed areas such as the head and neck, previous studies suggested that UV radiation may contribute to malignant transformation (2). Somatic mutations in cancer genomes are caused by multiple mutational processes, each of which generates unique combinations of mutation types termed "mutational signatures," which provide a powerful tool for identifying the cancer-causing agents (25). UV MSs, defined as a preponderance of C→T substitutions at dipyrimidine sites, including CC→TT, have been detected in the *TP53* tumor suppressor gene from squamous cell carcinoma (SCC), basal

cell carcinoma, precursors of SCC, and even normal sun-exposed skin (26, 27). A generally confirmed UV signature is defined as follows: ≥60% of mutations are C→T at dipyrimidine sites, with ≥5% CC→TT (27). The extracted MS data from pilomatrical tumors, however, showed <30% C→T changes (mostly associated with SBS1), with no predominant MS patterns, precluding UV radiation as a tumorigenic factor. SBS1 was the most prominent pattern in all pilomatrical tumors (Figure 2A). SBS1 mutations, associated with deamination of 5-methyl cytosine, are correlated with age and arise at different rates in different tissue types (28). Although SBS1 has not been implicated in specific causes of cancer, the distinct MS configuration in MP and BP1 with the *FGFR4* mutations suggests the existence of alterations in oncogenic pathways other than CTNNB1/Wnt/β-catenin signaling.

Loss of function in *TP53* plays a role in the acquisition of new genetic events contributing to malignant transformation in many cancers. In contrast to benign tumors, we observed complete loss of p53 only within MP with the *TP53* splicing variant; thus, inactivation of the p53 tumor suppressor is considered to be crucial in the final step of carcinogenesis. Currently, because we have no molecular methods for distinguishing MP from BP, p53 IHC could be useful in the diagnosis of malignancy. β-catenin IHC patterns also reflect innate *CTNNB1* status: in this study, nuclear and cytoplasmic β-catenin immunostaining corresponded to *CTNNB1* mutations in the control benign tumor, whereas normal membranous expression was detected in both MP and BP1, which harbored the oncogenic mutation in *FGFR4*. IHC studies could be very useful for predicting the underlying oncogenic pathways in pilomatrical tumors.

In summary, we have reported a novel *FGFR4* mutant and illustrated MS patterns in pilomatrical tumors. The results suggest the existence of a distinct tumorigenic pathway other than *CTNNB1*/Wnt/β-catenin signaling in these tumors. This is the first extensive genetic analysis of MP and BP through WES and MS analysis. Given the rarity of pilomatricomas and the lack of relevant genetic data, further work is necessary to confirm the incidence of this mutation and its clinical significance.

## Conflicts of Interest

No potential conflicts of interest are disclosed by the authors. The funders had no role in the design of the study or in the decision to publish the results.

## Authors' Contributions

Bae and Yeo had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Yeo; Acquisition, analysis, and interpretation of data: Bae; Drafting of the manuscript: Bae; Critical revision of the manuscript for important intellectual content: Bae and Yeo; Obtained funding: Bae and Yeo; Administrative technical, or material support: Yeo; Study supervision: Bae and Yeo.

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