Abstract

PURPOSE: Phyllodes tumors (PTs) of the breast are rare, accounting for less than 1% of all breast tumors. Among PTs, malignant PTs (MPTs) have malignant characteristics and distant metastases occur in about 20% to 30% of MPTs. However, there is no effective treatment for MPTs with distant metastasis, resulting in an abject prognosis. We performed targeted deep sequencing on PTs to identify the associations between genetic alterations and clinical prognosis.

METHODS: We performed targeted deep sequencing to evaluate the genetic characteristics of PTs and analyzed the relationships between clinical and genetic characteristics.

RESULTS: A total of 17 PTs were collected between 2001 and 2012. Histologic review was performed by pathologists. The samples included three benign PTs, one borderline PT, and 13 MPTs. The most frequently detected genetic alteration occurred in the TERT promoter region (70.6%), followed by MED12 (64.7%). EGFR amplification and TP53 alteration were detected in four MPTs without genetic alterations in MED12 and TERT promoter regions. Genetic alterations of RARA and ZNF703 were repeatedly found in PTs with local recurrence, and genetic alterations of SETD2, BRCA2, and TSC1 were detected in PTs with distant metastasis. Especially, MPT harboring PTEN and RB1 copy number deletion showed rapid disease progression.

CONCLUSIONS: In this study, we provide genetic characterization and potential therapeutic target for this rare, potentially lethal disease. Further large-scale comprehensive genetic study and functional validation are warranted.
Introduction
Phyllodes tumors (PTs) of the breast are rare, accounting for less than 1% of all breast tumors [1]. In contrast to invasive carcinoma of breast, PTs develop in the mesenchymal fibroepithelial tissues [2]. Based on the pathologic degree of stromal cellularity, atypia, stromal overgrowth, status of tumor border and mitoses, the World Health Organization categorized PTs as benign, borderline, and malignant [2,3].

Malignant phyllodes tumors (MPTs) frequently result in tumor recurrence and distant metastasis compared to benign and borderline PTs [1–3]. Approximately 20% to 30% of MPTs follow a distinctly metastatic disease course [2]. Treatment of MPTs is complete surgical excision, as for other breast cancers [4,5], and adjuvant radiation therapy has benefit in some cases of MPT [6]. MPTs with distant metastasis show considerable morbidity with rapid progression and are treated with chemotherapy. However, chemotherapy for metastatic MPTs is rarely effective, and these tumors have a dismal prognosis [1].

Recent genetic studies provided information on genetic alterations and therapeutic clues for MPT. Somatic mutation of mediator complex subunit 12 (MED12), the mediator complex between transcription factors and the RNA polymerase II initiation complex, was frequently observed in PTs [7]. Nearly all somatic mutations occurred in exon 2 of MED12 and were more frequently detected in benign PTs compared with MPTs [8,9]. In addition, MPTs have been reported to be associated with other somatic mutations in TP53, RB1, and EGFR [7].

In addition, next-generation sequencing revealed complex genetic alterations of PTs. Somatic mutation in TERT was reported to cooperate with MED12 alteration in PTs, and mutations in other genes including TP53, EGFR, NF1, CDKN2A, CDKN2B, and RARA were also observed [10,11]. In these studies, MPTs had more genetic alterations than benign and borderline PTs [7], but there was no difference in the number of mutations between primary and metastatic lesions [11].

In spite of comprehensive genetic studies of MPTs, associations between genetic alterations and clinical prognosis have not been revealed. Even though MPTs have malignant characteristics, distant metastasis does not occur in 80% of MPTs [1,2]. However, there is no effective treatment for MPTs with distant metastasis, and patients with these tumors have a dismal prognosis.

In this study, we performed targeted deep sequencing on MPTs and analyzed the relationships between genetic alterations and clinical characteristics, including prognosis.

Materials and Methods

Patients
This study involved prospective exploratory analysis of patients with PT of the breast at Samsung Medical Center and Seoul National University Hospital. Women who were diagnosed with PT of the breast by diagnostic examination and pathologic review and received curative surgery were enrolled. All patients provided written informed consent, and study approval was obtained from the Institutional Review Board of Samsung Medical Center, Seoul, Korea (IRB No: SMC 2017–04-073).

Pathologic Grade
Experienced pathologists reviewed all pathology specimens to determine benign, borderline, or malignant PT. They also described the following tumor characteristics: pathologic grade, stromal cellularity, atypia, stromal overgrowth, status of tumor border, mitosis per 10 high power fields (10HPF), tumor size, and necrosis.

DNA Extraction
Unstained sections (4 mm) of tumors consisting of more than 75% malignant cells were dissected under microscopy by comparison with an H&E-stained slide, and genomic DNA was extracted using a Qiagen DNA FFPE Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. After extraction, DNA concentration and 260/280-nm and 260/230-nm ratios were measured by spectrophotometry (ND1000, NanoDrop Technologies, Thermo-Fisher Scientific, MA). Each sample was then quantified using a Qubit fluorometer (Life Technologies, Carlsbad, CA). Libraries were prepared from samples with total genomic DNA yield >10 ng.

Targeted Deep Sequencing Using a Customized Cancer Panel (CancerSCAN™)
Genomic DNA (250 ng) from each tissue was sheared in a Covaris S220 ultrasonicator (Covaris, Woburn, MA) and used with CancerSCAN™ probes and a SureSelect XT reagent kit HSQ (Agilent Technologies) for construction of a library according to the manufacturer’s protocol.

This panel is designed to enrich exons of 381 genes (Supplementary Table 1), covering 366.2 kb of the human genome. After enriched exome libraries were multiplexed, the libraries were sequenced on a HiSeq 2500 sequencing platform (Illumina). Briefly, a paired-end DNA sequencing library was prepared through gDNA shearing, end-repair, A-tailing, paired-end adaptor ligation, and amplification. After hybridization of the library with bait sequences for 27 hours, the captured library was purified and amplified with an index barcode tag, and the library quality and quantity were assessed. Sequencing of the exome library was performed using the 100-bp paired-end mode of the TruSeq Rapid PE Cluster Kit and TruSeq Rapid SBS Kit (Illumina).

Variant Detection Using the Customized Cancer Panel
Sequence reads were mapped to the human genome (hg19) using Burrows-Wheeler Aligner (BWA) [12]. Duplicate read removal was performed using Picard and SAMtools [13]. Local alignment was optimized using the Genome Analysis Toolkit (GATK) [14]. Variant calling was performed only in regions targeted in CancerSCAN™. To detect single nucleotide variants, we integrated the results of three kinds of variant caller, which increased the sensitivity [15–17]. Pindel was used to detect indels [18]. Copy number variations were calculated for targeted regions by dividing the read depth per exon by the estimated normal reads per exon using an in-house reference.

Results

Clinicopathological characteristics
A total of 17 PTs were collected between 2001 and 2012 (Table 1). Histologic review by pathologists revealed three benign PTs, one borderline PT, and 13 MPTs. All patients were female, and the median age was 45.7 years (range 26.2–72.2). All patients underwent curative surgery, and two patients received adjuvant radiotherapy.

All specimens were primary breast PT tissues except for one metastatic lung tissue. Disease recurrence occurred in five patients;
three with local recurrence and two with lung metastasis (Supplementary Figure 1). One patient with lung metastasis received palliative chemotherapy and radiotherapy, but she died due to disease progression.

### Genetic Alterations in Phyllodes Tumors

Among 381 genes included, the most frequently detected site of genetic alteration was the TERT promoter region (12 of 17 cases, 70.6%) (Figure 1). The major alteration was a G>A transition in the promoter region (12 of 13 cases), and one intra-chromosomal translocation was detected. Genetic alteration of MED12 was also frequently detected (11 of 17, 64.7%). Nonsynonymous single nucleotide variants (SNVs) of MED12 were detected in seven PTs and non-frame shift indels in four PTs. Genetic alterations of TERT and MED12 were not mutually exclusive, and 10 cases of PT had genetic alterations in both genes.

Three benign PTs harbored both TERT promoter and MED12 genetic alterations, whereas other genetic alterations were not commonly observed. One borderline PT had only genetic alteration in the TERT promoter.

After MED12 and TERT, frameshift deletion of RAD50 was the most commonly detected genetic alteration, followed by SETD2

### Genetic Characterization of Malignant Phyllodes Tumor According to Tumor Recurrence

Of 12 MPTs, five experienced tumor recurrence (Table 1 and Figure 1). No recurrence was observed in benign and borderline PTs. Three cases were local recurrence, and two were pulmonary metastasis. All primary tumors with metastasis had genetic alteration of MED12 or TERT promoter.

Besides these two genes, all MPTs with local recurrence had genetic alteration of RARA, but other genetic alterations were not repeatedly observed. Two PTs with pulmonary metastasis had a similar genetic landscape of BRCA2 nonsynonymous SNV, SETD2 and TSCI genetic alterations. One PT had NRAS, PALB2, and PTCH2 SNVs, and the other had NCOI and PDGFRB SNVs and copy number deletions in PTEN and RB1.

Lastly, we compared genetic alterations between primary tissue and lung metastasis (SMC1411 and SMC1412). This analysis showed a similar genetic profile between the two samples, but more genetic alterations, including ASXL1, DNMT1, and ZNF217, were observed in the pulmonary metastatic lesion.

### Discussion

In this study, genetic alterations of TERT promoter and MED12 were the most frequently observed alterations in all subtypes of PT, as in previous genetic studies. We also found that EGFR amplification and/or TP53 alteration were possible driver mutations in PTs without TERT and MED12 alterations. Genetic alteration of RAD50, a DNA repair gene, was also frequently detected in PTs, and BRCA2 SNVs were only detected in MPT with recurrence. Breast mesenchymal fibroepithelial tumors comprise benign fibroadenomas and PTs. While fibroadenoma commonly occurs, PT is rarely detected [19]. Large scaled genetic studies for mesenchymal fibroepithelial tumors showed that MED12 was the most commonly mutated gene in both fibroadenomas and PTs [20,21]. Other genetic studies also reported that a MED12 mutation was one of the most common and ancestral genetic alteration in PT [8,22,23]. Among these studies, about 60% of fibroadenomas had a MED12 somatic mutation [21]. In terms of PTs, one showed that 70% of PTs had a MED12 genetic alteration regardless of histologic grade [22], and others reported that MED12 genetic alteration was more frequently detected in benign and borderline PTs compared with MPTs [8,20,23].

Recent comprehensive genomic profiling of MPTs using a next-generation sequencing technique showed that approximately 50% of MPTs had MED12 short variants [11]. Regardless of PT grade, 72% of PTs had MED12 short variants [20]. In this study, we sequenced the entire exons of MED12 and found that 7 of 12 MPTs had MED12 alterations. Especially, we could specify that 5 of 7 were nonsynonymous SNVs, and two were non-frame shift deletions. All genetic events of MED12 occurred in exon 2.
Figure 1. Genetic landscape of phyllodes tumors (N = 17).

Figure 2. Frequency of genetic alterations identified in phyllodes tumors (N = 17).
In addition to MED12, the TERT promoter was a repeatedly mutated region in PTs. A previous study reported that 11 of 18 tested malignant PTs had genetic variants in the TERT promoter [11], and another study showed that 60–70% of PTs had TERT genetic alterations [10]. Our study yielded similar results. All benign and borderline PTs and 7 of 12 MPTs had genetic alteration in the TERT promoter. In addition, all TERT genetic alterations were found together with MED12 variants with the exception of one MPT.

MPTs without TERT and MED12 genetic alterations had variant genetic alterations. EGFR amplification and TP53 and DNMT3A mutations were repeatedly observed, and we suggest that these mutations possibly initiate tumorigenesis in the absence of TERT and MED12 alterations. One malignant PT without any of the above genetic alterations had PIK3CA H1047R SNV. In a previous study, PIK3CA mutation was present in malignant PT without MED12 alteration [11,20]. They showed that genetic alterations of NFI, RB1, PIK3CA, EGFR and TP53 occurred in only borderline and malignant PTs [20]. Therefore, we suggest that this malignant PT might have similar genetic characteristics to invasive carcinoma of the breast. In terms of TP53, a previous study reported that mutations were frequently detected regardless of the status of MED12 or TERT [11,20]. However, we did not find TP53 co-mutation with MED12 or TERT promoter alterations.

Lastly, we attempted to identify genetic characteristics associated with tumor recurrence. Genetic alterations of RAR4 and ZNF703 [11] were repeatedly found in PTs with local recurrence, and genetic alterations of SETD2, BRCA2, and TSC1 were detected in PTs with distant metastasis. Previous genomic study of fibroepithelial tumor of breast already reported RARA, SETD2 and other genetic mutations being potential therapeutic targets [20]. But they did not consider clinical outcome of PTs. In our study, one case of MPT harboring PTEN and RB1 copy number deletion had rapid disease progression despite repeated metastasectomy and palliative chemotherapy and radiotherapy. Interestingly, targeted agents for these genetic alterations already exist. PARP inhibitor targeting BRCA2 and mTOR/RAK inhibitor for TSC1 would be potential target agents for the treatment of metastatic MPTs [24,25].

In this study, we performed genetic analysis of PTs using targeted deep sequencing. We also characterized the genetic aberrations of metastatic PTs, and the identified genetic alterations are candidate therapeutic targets of small molecules. In spite of the small sample size, our study provides insight into not only the genetic characterization, but also therapeutic guidelines for this rare and potentially lethal disease. Further large-scale comprehensive genetic studies and functional validation will provide a fundamental understanding of the genetic characteristics of phyllodes tumor and clues to effective therapeutic strategies.

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