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Mutational Profiling of Malignant Mesothelioma Revealed Potential Therapeutic Targets in *EGFR* and *NRAS*

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Abstract

Pemetrexed and platinum (PP) combination chemotherapy is the current standard first-line therapy for treatment of malignant mesothelioma (MM). However, a useful predictive biomarker for PP therapy is yet to be found. Here, we performed targeted exome sequencing to profile somatic mutations and copy number variations in 12 MM patients treated with PP therapy. We identified 187 somatic mutations in 12 patients (65 synonymous, 102 missense, 2 nonsense, 5 splice site, and 13 small coding insertions/deletions). We identified somatic mutations in 23 genes including *BAP1*, *TP53*, *NRAS,* and *EGFR*. Interestingly, rare *NRAS* p.Q61K and *EGFR* exon 19 deletions were observed in 2 patients. We also found somatic chromosomal copy number deletions in *CDKN2A* and *CDKN2B* genes. Genetic alteration related to response after PP therapy was not found. Somatic mutation profiling in MM patients receiving PP therapy revealed genetic alterations in potential therapeutic targets such as *NRAS* and *EGFR*. No alterations in genes with potential predictive role for PP therapy were found.

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Introduction

Malignant mesothelioma (MM) is a rare [1], highly malignant tumor that arises from mesothelial cells lining the serosal cavities of the body, including pleural, peritoneal, and pericardial surfaces. Despite treatment, the median survival of patients with MM currently ranges from 12 to 18 months from the time of diagnosis [2,3]. Previous retrospective studies have shown that prognosis of MM is associated with several clinical variables including performance status, sex, anemia, radiological parameters at presentation, and molecular or pathologic findings [4,5]. BRCA1-associated protein-1 (*BAP1*) mutations is known to be related to a high rate of MM and MM associated with germline *BAP1*mutations has a better prognosis of overall 7-fold increased long-term survival compared to sporadic MM [6].

Pemetrexed and platinum (PP) combination chemotherapy is the current standard first-line therapy for systemic treatment of MM. Response rate of PP therapy is approximately 40%; almost half of all patients are primary resistant, and all develop resistance ultimately [3,7].

Many studies have investigated the biology of mesothelioma in order to identify novel molecular therapeutic targets as well as potential predictive or prognostic biomarkers. MM may arise as polyclonal tumors, we need to evaluate simultaneously several different molecular targets in different MM cell clones, as each clone may carry its own distinct set of molecular alterations [8]. Before 2015, most studies used copy number arrays to profile potential chromosomal variations and Sanger sequencing methods to identify somatic mutations in tumors. Recently, genome wide somatic mutations of MM were profiled using next-generation sequencing (NGS) methods with whole genome [9,10], whole exome

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[11–15] and targeted amplicons [16]. However, to the best of our knowledge, predictive biomarkers for PP therapy has not been identified in MM patients. In the present study. We performed targeted exome sequencing to profile somatic mutations and copy number variations in malignant pleural and peritoneal mesothelioma patients treated with PP therapy.

Materials and Methods

Patients and Tissue Samples

Between January 1990 and December 2012, 98 MM patients were diagnosed and received treatment at Asan Medical Center in Seoul, Korea. The diagnosis of MM was based on standard histological and immunohistochemical criteria. Of 98 patients, 71 received systemic chemotherapy and 51 of these received first-line PP therapy. Thirty-two patients treated with PP therapy as first-line palliative chemotherapy had tissues available as formalin fixed, paraffin embedded (FFPE) blocks. Hematoxylin/eosin stained (H&E) slides and corresponding FFPE blocks were reviewed by a pathologist, who selected the area with tumor cells for genomic DNA extraction. Fifteen patients with adequate tumor tissues were included for targeted NGS. Targeted NGS was performed with samples from 15 patients with adequate tumor tissues, but 3 samples did not pass quality control.

Genomic DNA Extraction

After review of the matched H&E slides for each FFPE tissue section by a pathologist, 2 to 5 sections (6 μm thick) were used for extraction of genomic DNA for each FFPE tissue, depending on the sample size and cellularity. After treatment with xylene and ethanol for de-paraffinization, genomic DNA was isolated using a NEXprep FFPE Tissue kit (#NexK-9000; Geneslabs, Korea), according to the manufacturer's protocol. Briefly, the tissue pellet was completely lysed by incubation with proteinase K in lysis buffer overnight at 56°C, followed by additional incubation for 3 minutes with magnetic beads and solution A at room temperature. After incubation for 5 minutes on a magnetic stand, supernatant was removed and washed three times with ethanol. After air-drying the beads for 5 minutes, DNA was eluted in 50 μL of nuclease-free water, and quantified using a Quant-iT^{TM} PicoGreen dsDNA Assay kit (Invitrogen, Carlsbad, CA, USA).

Targeted Next Generation Sequencing

Targeted NGS was performed using the MiSeq platform (Illumina, San Diego, CA, USA) with OncoPanel version 2 (OPv2) for capturing exons of 505 cancer-related genes plus partial introns from 15 genes often rearranged in cancer [17]. gDNA 200 ng was fragmented by sonication (Covaris Inc., Woburn, MA) to an average of 250 bp, followed by size selection using Agencourt AMPure XP beads (Beckman Coulter, High Wycombe, UK). A DNA library was prepared by ligation of 50 ng of purified DNA with a TruSeq adaptor using a SureSelect XT Reagent kit (Agilent Technologies, Santa Clara, CA), Each library was synthesized with sample-specific barcodes of 6 bp, quantified using PicoGreen, and four libraries were pooled to a total of 600 ng for hybrid capture using an Agilent SureSelectXT custom kit (OPv2 RNA bait, 2.9 Mb; Agilent Technologies). The concentration of the enriched target was measured by quantitative polymerase chain reaction (PCR) (Kapa Biosystems, Woburn, MA), and loaded on the MiSeq platform (Illumina Inc., San Diego, CA) for paired end sequencing.

Bioinformatics Analysis

Sequenced reads were aligned to the human reference genome (NCBI build 37) with BWA (0.5.9) with default options [18]. To remove PCR duplicates from the aligned reads, we used the MarkDuplicates of Picard package (available at http://broadinstitute.github.io/picard). De-duplicated reads were re-aligned at known indel positions with the GATK IndelRealigner [19]. Base qualities were then recalibrated using the GATK TableRecalibration. Somatic single nucleotide variants and short indels were detected with an unmatched normal using Mutect (1.1.6), VarDict and SomaticIndelocator in GATK [19–21]. Common and germline variants from candidates of somatic variants were filtered out with common dbSNP (141 found in >1% of samples), Exome Aggregation Consortium (ExAC; r0.3.1), Korean Reference Genome database (KRGDB) and in-house panel of normals [22,23]. Final somatic variants were annotated using Variant Effect Predictor (version 79) [24] and were then converted to maf file format using vcf2maf (https://github.com/mskcc/vcf2maf). False-positive variants were manually curated using Integrative Genomics Viewer (IGV) [25]. Both somatic mutations and copy number variations were loaded in a local cBioPortal [26,27]. Pathway analysis was performed using DAVID [28].

Structural Variation Analysis

Copy number analysis was performed using CNVkit, and copy numbers of tumors were analyzed against a panel of unmatched normals [29]. Heatmap plots were generated using the heatmap command from the CNVkit with segments files. The GISTIC algorithm was applied to samples that satisfied the quality criteria to identify significant focal and arm level amplifications and deletions [30]. The GISTIC q-value cut-off was set to 0.25.

Rearrangement analysis was performed using BreaKmer and candidates of germline mutations or false positives were filtered out with an in-house panel of normals [31].

Statistical Analysis

Overall survival (OS) was defined as the time from chemotherapy initiation until death or when the patient was last known to be alive. Progression-free survival (PFS) was defined as the time from

Table 1. Patient Characteristics and Clinical Outcomes of PP Therapy (N = 51)

	No. of Patients	% of Patients
Age		
Median (range)	58 (36-75)	
Sex		
Female	21	41.2
Male	30	58.8
Primary site		
Peritoneum	21	41.2
Pleura	28	54.9
Pleura and pericardium	2	3.9
Cytoreductive surgery		
Not performed	33	64.7
Performed	18	35.3
Best overall response of PP therapy		
CR	3	5.9
PR	9	17.6
SD	27	52.9
PD	9	17.6
Not available	3	5.9

PP; pemetrexed and platinum, CR; complete response, PR; partial response, SD; stable disease, PD; progressive disease.

chemotherapy initiation to the first confirmation of progressive disease or death. Survival curves of PFS and OS were plotted using the Kaplan-Meier method. R package (http://www.R-project.org/) was used to perform *t* tests.

Results

Patient Characteristics and Clinical Outcomes of PP Therapy

Between January 1990 and December 2012, 51 patients received PP combination therapy as first-line chemotherapy at Asan Medical center. The median age was 58 years (range, 36–75 years) and 30 patients (58.8%) were males. The primary site of malignant mesothelioma was pleura in 28 patients, peritoneum in 21 patients, and pleura and pericardium in 2 patients. Among the 51 patients, 33 underwent cytoreductive surgery (Table 1) and 44 were evaluated for tumor response. The best overall tumor responses were complete response (CR) in 3 patients, partial response (PR) in 9 patients, and stable disease (SD) in 27 patients. The median PFS was 7.5 months (95% CI, 3.9-11.0 months), and the median overall survival (OS) was 17.8 months (95% CI, 9.6-26.0 months, Figure 1).

Out of the 51 patients, 15 patients with adequate tumor tissues were included for targeted next-generation sequencing (NGS), but 3 samples did not pass quality control. Finally, samples from 12 patients were sequenced, and their patient characteristics, clinical outcomes, and the results of the bioinformatics analysis are shown in Table 2. Out of the 12 patients, 4 showed short PFS less than 12 months; particularly, 2 patients had progressive disease immediately after PP therapy and showed less than 2 months of PFS. In contrast, 8 patients showed prolonged PFS of over 12 months.

Landscape of Somatic Mutations in MM

We performed targeted capture sequencing (OncoPanel v2, OPv2; Supplementary Table 1) on tumor samples from 12 cases of MM. These satisfied our minimum quality control criteria (mean target coverage ≥90x, target bases over 30x ≥80%). On average, 12,050,189 reads were generated for each sample, yielding coverage of targeted regions to a mean depth of 136x. More than 90% of the targeted regions were sufficiently covered for confident mutation calling (≥30 reads) (Supplementary Table 2).

We identified a total of 32 somatic mutations in coding regions, including 7 synonymous mutations, 19 missense mutations, 2 splice site mutations, and 4 small coding insertions/deletions (indels) (Table 3) in 9 cases (no mutation detected in 3 cases). We detected a mean of 2.8 somatic mutations per tumor (range, 1–8), corresponding to an average of 1.4 mutations per megabase (range, 0.5–4; Figure 2A). The mutational spectrum in our 9 MM cases was dominated by C>T transitions, which is in line with the results from a previous mesothelioma study [13].

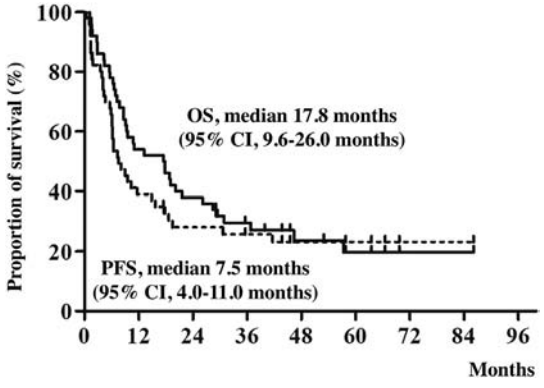

Figure 1. Overall survival and progression-free survival of patients who received Pemetrexed and Platinum therapy.

Table 2. Patient Characteristics and Clinical Outcomes of 12 Patients with Bioinformatics Analysis

Case	Age/Sex	Primary Site	CRS	Best Overall Response to PP	PFS, Months
1	63/F	peritoneum	Performed	SD	17.6
2	61/F	pleura	Performed	CR	69.7
3	58/M	peritoneum	Performed	SD	51.3
4	47/M	pleura	Performed	PR	15.7
5	52/M	pleura	Performed	SD	6.1
6	56/M	pleura	Not performed	PR	41.6
7	58/F	peritoneum	Not performed	SD	55.5
8	54/M	peritoneum	Performed	SD	6.4
9	36/F	pleural and pericardium	Performed	SD	23.4
10	53/M	pleura	Performed	PD	1.4
11	49/F	pleura	Not performed	PD	1.6
12	51/M	pleura	Not performed	SD	17.5

CRS; cytoreductive surgery, PP; pemetrexed and platinum, CR; complete response, PR; partial response, SD; stable disease, PD; progressive disease, PFS; progression free survival.

Recurrently Mutated Genes

We found 23 genes harboring protein-altering mutations, 2 of which were recurrently mutated in at least 2 individuals (Figure 2A). The genes that most frequently carried somatic mutations were *BAP1* and *TP53* (2 out of 12 cases (16.7%)). In detail, we found a somatic mutations in *BAP1*, which resulted in p.Q85R and p.S469Rfs*22 amino acid change (peritoneum –1; pleural –1; Figure 2B). Somatic mutations in *TP53* were also found at the P53 DNA binding domain

Table 3. Non-Silent Somatic Mutations Identified in 12 Malignant Mesotheliomas

Gene	Variant Class	Reference	Variant	A.A Change
APC	Missense mutation	G	A	R106H
ATR	Missense mutation	G	A	A2575V
BAP1	Missense mutation	T	C	Q85R
CREBBP	Missense mutation	G	C	P2077A
CUBN	Missense mutation	G	A	A99V
DICER1	Missense mutation	A	C	F72C
DLC1	Missense mutation	C	G	D1406H
FGFR1	Missense mutation	T	C	D175G
FGFR4	Missense mutation	G	A	V168I
GNAS	Missense mutation	G	A	D883N
IGF1R	Missense mutation	A	G	T917A
KMT2D	Missense mutation	G	A	S5404F
NF1	Splice-site mutation	G	A	X2580_splice
NRAS	Missense mutation	G	T	Q61K
PAK7	Missense mutation	C	T	D421N
PATZ1	Missense mutation	A	C	L195W
PBRM1	Missense mutation	T	C	N1115D
PTCH1	Missense mutation	C	A	W851L
ROBO2	Missense mutation	C	T	R689C
TP53	Missense mutation	T	C	Y205C
TP53	Splice-site mutation	T	C	X261_splice
PREX2	Frame shift insertion	-	A	P1365Tfs*20
KDM5C	Frame shift deletion	G	-	Q859Rfs*2
EGFR	Inframe shift deletion	GGAATTAA GAGAAGC	-	E746_A750del
BAP1	Fram shift deletion	CG	-	S469Rfs*22

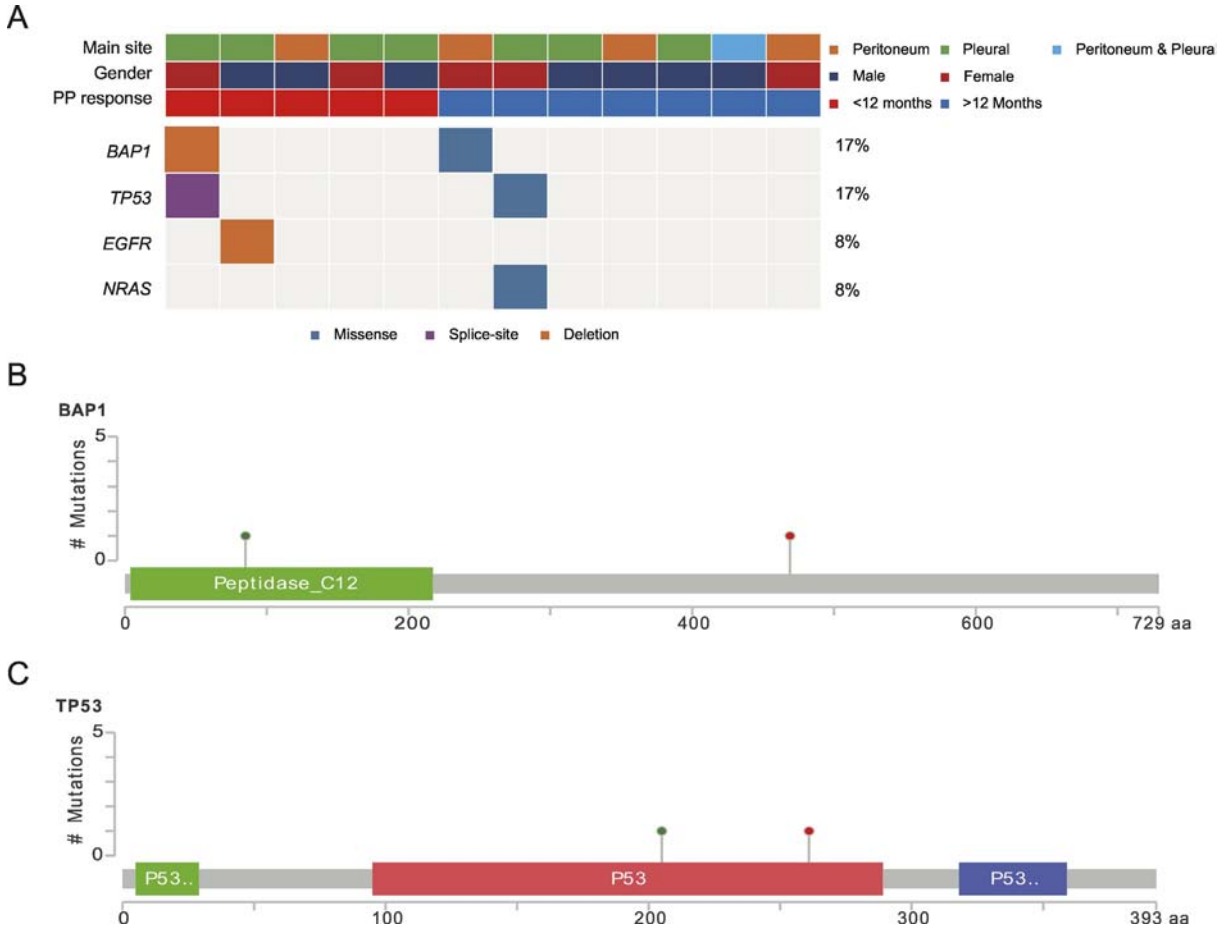

Figure 2. Mutational landscape in 12 malignant mesotheliomas.(A) Clinical data (main site, sex, Pemetrexed and Platinum therapy (PP) response) and mutation type for 21 genes that were recurrently mutated in 12 malignant mesotheliomas. (B) Mutation diagram of *BAP1*. (C) Mutation diagrams of *TP53* (green color represents missense mutation; red color represents frame-shift deletion).

(p.Y205C and p.X261_splice) (Figure 2C). These two TP53 mutations were observed in pleural mesothelioma. No noticeable genetic alteration was observed in tumors that rapidly progressed after PP therapy, or in tumors with long lasting durable response after PP therapy. Therefore, we did not find any genetic alteration related to treatment response after PP therapy.

Previous studies of MM have reported significantly or recurrently mutated genes such as *BAP1*, *TP53*, *CUL1*, *NF2*, *TP53*, *KIT*, and *MET* [9–16]. However, our 9 MM cases had relatively lower mutational frequencies for these genes (22%, 22%, 0%, 0%, 0%, and 0%, respectively).

Structural Variations

We profiled the somatic copy number variations (CNVs) of 12 malignant mesotheliomas using targeted NGS (OPv2) data obtained by CNVkit, and identified *CDKN2A* and *CDKN2B* copy number deletions. We performed GISTIC2 analysis to detect significant focal CNVs, which yielded 2 amplified (9q34.12 and 17q21.3) and 1 deleted (9p21.3) regions (q-value <0.25; Figure 3, Supplementary Table 3). The most common recurrent focal amplification contained *ABL1* (9q34.12) and *COL1A1* (17q21.33). A focal deletion (9p21.3) seemed to target *CDKN2A*, *CDKN2B* (Supplementary Figure 1), which is a widely reported genomic alteration in MM [32].

We performed rearrangement analysis using targeted exome sequencing designed for detecting 15 recurrently rearranged genes in cancer, including *RET*, *ALK*, *BRAF*, and *AKT*. No rearrangement event was identified in any of our 12 MM cases.

Potential Therapeutic Candidates

Interestingly, we found one missense mutation and one deletion that may be potential therapeutic targets for MM. A missense *NRAS* mutation was identified at amino acid of 61 position (p.Q61K) in malignant pleural mesothelioma (Figure 4A and B). A novel *EGFR* exon 19 deletion (p.E746_A750del) was detected in malignant pleural mesothelioma (Figure 4C and D). These two different somatic mutations were observed in separate patients.

Discussion

We identified 32 somatic mutations in 12 MM patients treated with PP therapy using targeted exome sequencing. We identified somatically recurrent mutations in 2 genes including *BAP1* and *TP53*. We also found somatic chromosomal copy number deletions in *CDKN2A* and *CDKN2B* genes. The results are similar to recent NGS studies on MM [9–16]. We did not find any noticeable genetic alteration related to PP therapy response.

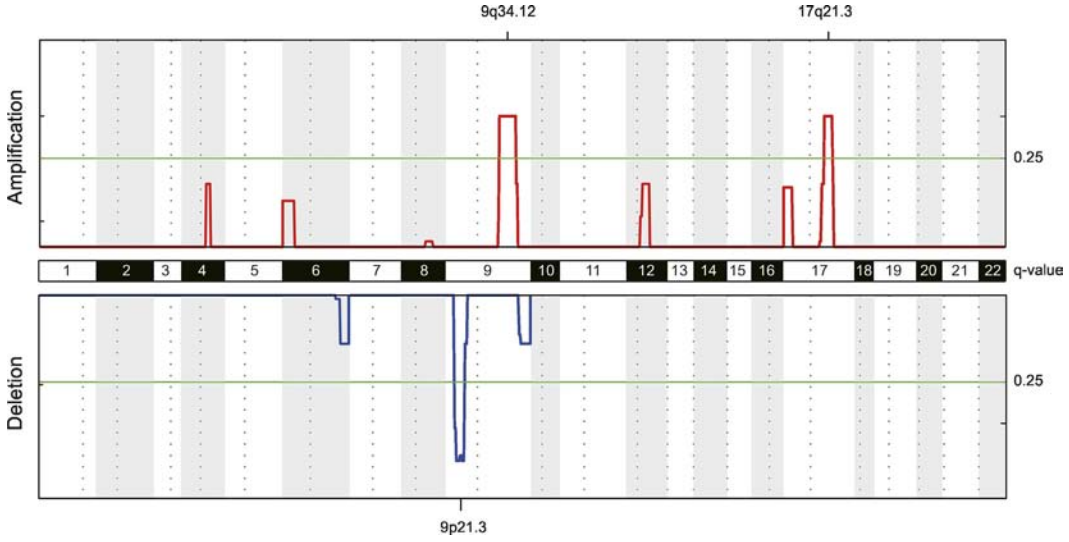

Figure 3. Copy number variants in 12 malignant mesotheliomas. GISTIC analysis identified 4 significantly altered copy number variations (q-value cutoff: 0.25).

Recently, genomic mutation profiling studies using NGS have provided valuable understanding of the genetic basis of MM [9–16]. These previous studies confirmed the genetic changes in *BAP1*, *TP53*, *CUL1, NF2,* and *LATS1-PSEN1* fusion. The frequency of somatic mutations in *BAP1* is approximately 40% in pleural MM [13]. However, somatic mutations in *BAP1* were relatively low in our study. In recent study, Nasu et al. [33] reported that approximately 60% of MM specimens had somatic mutations in *BAP1* and half of

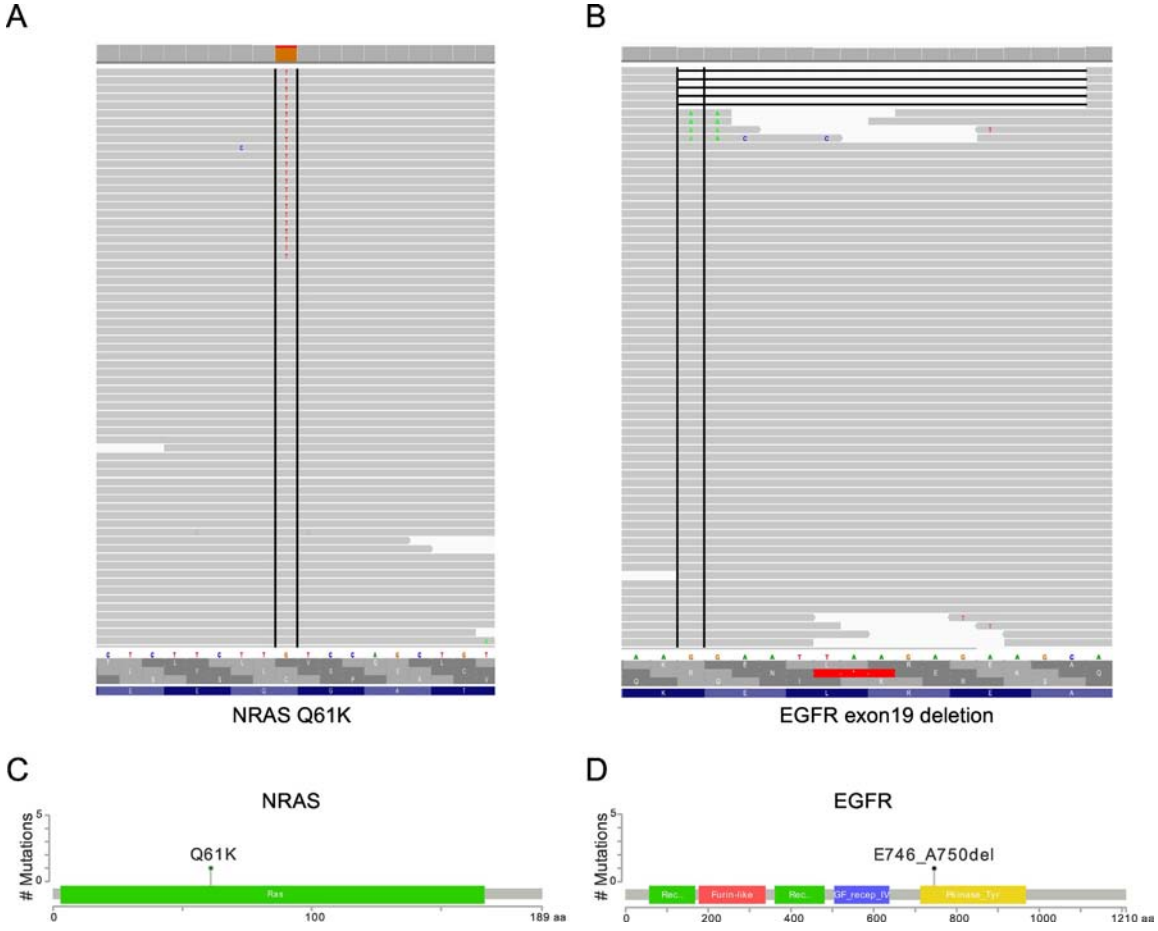

Figure 4. Somatic mutations in *NRAS* and *EGFR* as a potential therapeutic target. (A) *NRAS* Q61K mutation in malignant pleural mesothelioma shown by integrative genomic viewer (IGV). (B) *EGFR* exon 19 deletion in malignant pleural mesothelioma, shown by IGV. (C) Mutation diagram of *NRAS* Q61K. (D) mutation diagram of *EGFR* exon19 deletion.

the mutations were deletions which were too large to be detected using NGS (ranging from 300 to about 3,000 kb). We assume that these large deletions may affect relatively low frequency of somatic mutations in *BAP1* in this study.

TP53 is well-known tumor suppressor gene in multiple tumor types. Somatic mutations in *TP53* have been reported as one of the significantly mutated gene in MM. The frequency of somatic mutations in *TP53* is approximately 16% in MM (TCGA). Most of the somatic mutations in *TP53* were observed at recurrent hotspots such as K132N, R273H, V216L, C238F, G244D, G245S, Y234C, R273C, Q331*, A276D, and Q331*. Similarly, somatic mutations in *TP53* were detected in17% of MM in our study.

Notably, the rare, previously unreported *NRAS* Q61K mutation and *EGFR* exon 19 deletion may be potential therapeutic candidates in MM. This is the first report of *NRAS* mutation in mesothelioma. *NRAS* is an oncogene encoding a family of GDP/GTP-regulated switches and is frequently mutated in a diverse type of cancers such as melanoma and thyroid cancer. *RAS* gene family has a recurrently mutated hotspot at G12, G13 and Q61 amino acid positions. *NRAS* Q61K mutation is involved in the onset and progression of several cancers such as melanoma, papillary thyroid, colorectal, and ovarian tumor, and is often associated with poor prognosis [34]. Mutated *NRAS* triggers the MAPK signaling cascade through activation of RAF, which in turn activates MEK, thereby triggering ERK phosphorylation and cellular proliferation. Blocking a downstream signaling partner is an effective therapeutic strategy [35]; particularly, MEK inhibitor single therapy or combination therapy of MEK inhibitor with PI3K-AKT-mTOR inhibitors were shown to be effective in melanoma [36].

EGFR is a well-known oncogene that encodes a tyrosine kinase receptor. Activating somatic mutations in *EGFR* have been observed in 10–30% of non-small cell lung cancer (NSCLC) patients, and are used as genomic biomarkers for prediction of sensitivity to EGFR inhibitors in NSCLC. *EGFR* exon 19 (729-761 amino acid) deletions occur in approximately 50% of NSCLC patients and are well-characterized as a EGFR-tyrosine kinase inhibitor (TKI)-sensitive mutations. Interestingly, these mutations have not been reported in previous MM studies. In our current study, *EGFR* exon 19 deletion (p.E746_A750del) was identified in malignant pleural mesothelioma, which suggests that EGFR TKI could be a potential therapeutic candidate for MM by targeting *EGFR* exon 19 deletion.

Our study has the following limitations. First, only 12 tumor samples were included in mutation profiling using NGS. The small number of patients was due to the rarity of mesothelioma and the timescale of PP therapy. Using formalin fixed, paraffin embedded (FFPE) samples further limited the availability of adequate specimens for NGS. Second, because we collected clinical information in a retrospective manner, we could not collect the patients' history of smoking and occupational exposure to asbestos, which are risk factors for the development of mesothelioma. A previous study reported that asbestos exposure is associated with mutations in *KRAS* and worse prognosis in MM patients [37]. Lastly, we could not perform tumor-matched normal pair analysis due to limited sample availability.

Our study is the first to use targeted NGS to describe the somatic mutation profiles in malignant pleural and peritoneal mesothelioma treated with PP therapy. We did not find any predictive marker for PP therapy, but found potential actionable targets such as *NRAS* p. Q61K mutation and *EGFR* exon 19 deletion. Further investigations with a larger number of patients including functional studies for potential therapeutic targets with NRAS and EGFR inhibitors are needed.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tranon.2018.01.005.

Author Contributions

DK analyzed the data. JEK, YSH, KPK, DHL, SWK, SMC, SJJ, and TWK prepared the samples and generated the data. JEK and TWK designed the study. JEK and DK prepared the manuscript. All authors read and approved the final manuscript.

Competing Interests

The authors declare no conflict of interest.

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