Overview and Clinical Significance of Multiple Mutations in Individual Genes in Hepatocellular Carcinoma

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Abstract

Background

Multiple mutations (MMs) within individual oncogenes have been newly characterized as a mechanism for promotion of carcinogenesis. We investigated the spectra of the MMs and the clinical significance in hepatocellular carcinoma (HCC).

Methods

Whole-exome sequencing and gene expression profiling were performed in 223 surgically resected HCCs.

Results

MMs within individual genes was identified in 178 samples (79.8%, MMs tumors). All the remaining samples carried single mutation (20.2%, SM tumors). Mutations identified as MMs show different mutational patterns with higher functional impact compared with mutations identified as SM. Recurrence-free survival was significantly worse in the group with MMs tumors than the group with SM tumors ($P = 0.012$). MMs tumor was identified as an independent predictor for worse prognosis (hazard ratio, 1.72; 95%, $P = 0.045$). MMs were observed particularly in *MUC16* (15% of samples with at least one mutation in the gene) and *CTNNB1* (14%). Although there was no significant difference in *MUC16* mRNA expression between *MUC16* wild-type and *MUC16* SM tumors, the expression in *MUC16* MMs tumors was significantly enhanced compared with *MUC16* SM tumors ($P < 0.001$). MMs in *MUC16* were associated with viral hepatitis, higher tumor markers and vascular invasion. Recurrence-free survival was significantly worse in the *MUC16* MMs group than the *MUC16* SM group ($P = 0.022$); no significant difference was observed between the *MUC16* SM group and *MUC16* wild-type group ($P = 0.324$).

Conclusions

MMs are relatively common driver events that selectively occur in specific oncogenes and function in tumor-promoting activity.

Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death worldwide [1]. Advances in next-generation sequencing have enabled the examination of cancer genomes and led to the discovery of driver alterations. In HCC research, these advances have enabled the processing of the HCC genome, and somatic mutations, structural alterations, HBV integration [2], RNA editing and retrotransposon changes [3] have been identified. Somatic mutation in the *TERT* promoter has been identified as the most frequent alteration (approximately 60%) in HCC [4]. In coding regions, whole-exome sequencing detected frequent mutations in candidate driver genes, such as *TP53* (31%), *ARID1A* (28.2%), *CTNNB1* (18.8%), *MTDH* (14.7%), *AXIN1* (14.2%), *CDKN2A* (11.7%), and *ARID2* (10.9%) [3]. These comprehensive genomic analyses have already identified many pathways as potential therapeutic targets. However, most of the genetic alterations identified were shown to occur with low frequency. Thus, these findings indicate that HCC might be a disease for which the development of molecular targeted treatment is challenging.

Multiple mutations (MMs) in the same oncogene have been newly characterized as one of the mechanisms for the promotion of carcinogenesis [5]. Although tumour suppressor genes (TSGs) are known to be affected by multiple loss-of-function mutations [6, 7], no study has investigated MMs arising in the same oncogene during cancer initiation and development in a structured and consistent way. There MMs in the same oncogene were reported to be more frequent than expected: 9% of pan-cancer samples with at least one mutation in a oncogene harbored MMs. MMs confered enhanced oncogenicity in combination. These findings indicated that oncogenic MMs are a relatively common driver event, providing the underlying mechanism for clonal selection of suboptimal mutations that are individually rare but collectively account for a substantial proportion of oncogenic mutations. The previous report [5] was a pan-cancer analysis to overview the landscape of MMs in the same oncogene. Therefore, the frequency and clinical significance of MMs in individual cancer types remain unclear.

Here, we performed comprehensive genetic profiling of HCCs using whole-exome sequencing (WES) and gene expression profiling (GEP) analysis in a large Japanese population. To overview MMs in HCCs and assess the clinical relevance of MMs in HCC patients, we investigated the accumulation of MMs in each gene and the association between MMs and clinicopathological information.

Materials And Methods

Ethics statement

To investigate the biological characteristics of cancer and diathesis of each patient with cancer, the Shizuoka Cancer Center started Project High-tech Omics-based Patient Evaluation (HOPE) in 2014 [8]. Project HOPE was designed according to the “Ethical Guidelines for Human Genome and Genetic Analysis Research” revised in 2013 [8]. Written consent was obtained from all patients participating in Project HOPE. The present study used the data from Project HOPE and was approved by the Institutional Review Board of Shizuoka Cancer Center (approval no. 25–33). The study protocol conforms to the ethical guidelines of the Declaration of Helsinki.

Patient selection and study design

From January 2014 to March 2019, 223 HCCs were analyzed in Project HOPE. All tumor tissues were pathologically diagnosed as HCC. Tumor tissue samples were dissected from fresh surgical specimens. The surrounding normal tissue was also obtained whenever possible. In addition, peripheral blood was collected as a control for WES. DNA was extracted from tissue samples using a QIAamp DNA Blood MINI Kit (Qiagen, Venlo, The Netherlands). For RNA analysis, tissue samples were submerged in RNAlater solution (Thermo Fisher Scientific), minced and stored at 4°C overnight before RNA extraction. To validate our findings in other cohort, mutation profiles were extracted from the public database in the TCGA project [9].

WES analysis of HCC tissues using next-generation sequencing

WES analysis was performed as previously described [10, 11]. Briefly, DNA was subjected to WES on an Ion Proton System (Thermo Fisher Scientific). The sequence data derived from blood samples were used as matched controls. Mutations fulfilling at least one of the following criteria were discarded as false positive: (1) quality score < 60, (2) depth of coverage < 20, (3) variant read observed in one strand only, (4) clipped sequence length < 100 (avg_clipped_length < 100), (5) variant located on either sequence end (avg_pos_as_fraction < 0.05), and (6) mutation matches one on an in-house false-positive list. Parameters specified in criteria (4) and (5) were calculated by bam-readcount with option “-q 1” (ver. 0.8.0) (https://github.com/genome/bam-readcount).

GEP using DNA microarray analysis

GEP analysis was performed as previously described [10, 12]. Total RNA was extracted using an miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA samples with an RNA integrity number of greater than or equal to 6 were used for DNA microarray analysis. Labeled samples were hybridized to a SurePrint G3 Human Gene Expression 8 × 60 K v2 Microarray (Agilent Technologies, Santa Clara, CA, USA). The fold change between tumor and normal tissues from the same patient was calculated from the normalized values.

Construction of a catalogue of cancer-related genes

The classification for oncogenes and TSGs were obtained from COSMIC Cancer Gene Census [13], and OncoKB Cancer Gene List [14] as previously described, in our analysis pipeline Shizuoka Multi-omics Analysis Protocol[15].

CCLE cell line data

The mutation call data (depmap_19Q1_mutation_calls_v2.csv) for 1,601 cell lines and drug-sensitivity data (v17.3_fitted_dose_response.xlsx) for 1,065 cell lines were obtained from the DepMap (https://depmap.org/portal/), CCLE (https://portals.broadinstitute.org/ccle/) and Genomics of Drug Sensitivity in Cancer (GDSC; https://www.cancerrxgene.org/) databases.

Statistical analysis

Continuous variables were expressed as the median with IQR and compared using the Mann–Whitney *U* test. Univariate analysis for categorical variables was performed by the chi-square test and Fisher's exact probability test. The thresholds generally used in clinical settings were employed as the cut-off value of continuous variables for statistical processing. The overall survival (OS) and relapse-free survival (RFS) were calculated using the Kaplan–Meier method, and the log-rank test was used to evaluate the statistical significance of the differences. A Cox proportional hazard regression analysis was used for the multivariate prognostic analysis. All statistical analyses were performed using the JMP software package, version 14.0 for Mac (SAS Institute Inc., Cary, NC, USA). A *P*-value lower than 0.050 was considered statistically significant.

Results

Tumor samples and patients

In total, 223 HCC tissues were analyzed. The median patient age was 71 years old (IQR: 65–77 years). Small tumors tended to be excluded from Project HOPE, since the removal of tumor tissue samples in patients with small tumors would make their pathological diagnosis difficult. The median tumor size was 35 mm (range: 24–70 mm). The median follow-up period was 34.1 months; the 3-year OS was 81.5% and the median RFS after surgery was 27.6 months.

Overview of MMs in individual genes

In the 223 samples, MMs within a oncogene were identified in 35 (15%) samples and MMs within a TSG were identified in 29 (12%) samples. For all genes, MMs within individual genes was identified in 178 samples (79.8%,

MM tumors). All the remaining samples carried single mutation (20.2%, SM tumors, **Fig. 1a**). To compare the impact of genomic variant annotations and functional effect between mutations identified as SM and mutations identified as MMs, genomic variant were classified by SnpEffs into four levels in accordance with the variety of alterations as follows: high: nonsense mutation, frame-shift mutation and splice site mutation; moderate: missense mutation and in-frame indel; low: synonymous mutation; and modifier: untranslated region mutation. Mutations identified as MMs showed a higher fraction of high impact mutations than mutatios identified as SM; a larger impact on the protein structure caused by amino acid alterations were found in mutations identified as MMs than in mutations identified as SM (**Fig. 1b**). Furthermore, we evaluated the correlation between tumor mutation burden (TMB) and mutational signatures of the COSMIC database and MMs using deconstructSigs [16]. **Supplementary Fig. 1** shows MMs, TMB, and signature contributions in samples with mutation count of >50. The TMB was significantly higher in MMs tumors than that in SM tumors (**Fig. 1c**). As shown in **Fig. 1d**, three signature scores were significantly varied betweem MMs tumors and SM tumors. To assess the clinical impact on the presence of MMs in HCC, we performed prognostic analysis according to the presence of MMs. The RFS was significantly worse in the group with MMs tumors than in the group with SM tumors ($P = 0.012$, **Fig. 1e**). To consider the potential confounding of TMB with MMs, the prognostic analysis included the TMB. The distribution of TMB is shown in **Supplementary Fig. 2**. The cutoff value was set to 6.65 as 95% tile. The Cox proportional hazard analysis for RFS of all 223 patients who underwent resection identified MMs as an independent predictor for prognosis (hazard ratio, 1.72; 95% confidence interval, 1.01–3.17; $P = 0.045$) and showed that microvascular invasion ($P < 0.001$) was an independent factor that predicted poor survival (**Table 1**). No significant prognostic effect was found in the TMB.

Frequent MMs in a variety of oncogenes in HCC

Fig. 2a shows the number of mutated samples and the fraction of samples with MMs for 14 genes with 20 or more mutated samples in the present cohort (n = 223). MMs were frequently observed across a wide variety of genes; we found that 5% or more of the mutated samples carried MMs across 26 genes, particularly in *MUC16* (15% of samples with at least one mutation in the gene) and *CTNNB1* (14%). Correlations between MMs in *CTNNB1* (**Fig. 2b**) and *MUC16* (**Fig. 2c**) and TMB were investigated. In both genes, significant differences in the TMB were found between samples with SM and samples with the wild-type gene (*CTNNB1*, $P < 0.001$; *MUC16*, $P = 0.001$), but no significant differences were found between SM and MMs (*CTNNB1*, $P = 0.710$, *MUC16*, $P = 0.531$). Therefore, we evaluated the mutational pattern of MMs in the genes. Using deconstructSigs [16], mutational signatures of the COSMIC database were investigated. The liver-cancer-specific signature 16 [17] was significantly higher in samples with MMs in *CTNNB1* than samples with the wild-type *CTNNB1,* although no significant differences in the signature score between samples with SM in *CTNNB1* and samples with the wild-type *CTNNB1* (**Fig. 2d**). No significant differences in the signature 16 score between samples with MMs in *MUC16* and samples with the wild-type *MUC16* and between samples with SM in *MUC16* and samples with the wild-type *MUC16* were confirmed (**Fig. 2e**). The distribution of mutations and fraction of MMs for each position in *CTNNB1* and *MUC16* are shown in **Supplementary Fig. 3.** In *CTNNB1*, most mutations were located in major hotspots of exon 3. No significant difference of the frequency was observed between *CTNNB1* SM tumors and *CTNNB1* MMs tumors. In *MUC16*, mutations frequently located at exon 3 and there was no significant difference in the frequency between *MUC16* SM and *MUC16* MMs tumors. We investigated the allelic configuration of MMs by phasing from WES reads, which revealed that most MMs (83%) in *CTNNB1* were present in cis. While all of the MMs in *MUC16* was not located in a same amplicon, therefore the allelic configuration of MMs in *MUC16* could not be investigated in the present study. Next, we investigated the impact of MMs on gene expression in *CTNNB1*

(**Fig. 2f**) and *MUC16* (**Fig. 2g**). In *MUC16*, MMs had larger alterations of gene expression; although there was no significant difference of expression between samples with wild-type *MUC16* and samples with SM in *MUC16*, the expression in samples with MMs in *MUC16* was significantly enhanced compared with samples with SM in *MUC16* ($P = 0.047$). These results suggest that individually suboptimal mutations can confer enhanced oncogenic potential in combination as MMs. Based on the findings, for further investigation, we focused on *MUC16* as a candidate oncogene to validate the impact of MMs.

Functional relevance of MMs in oncogenes

To assess the impact of MMs on phenotypes in cancer cell lines, an analysis of drug sensitivity screens in Cancer Cell Line Encyclopedia (CCLE) cell lines [18] was performed. Box plots (**Supplementary Fig. 4**) show sensitivity to regorafenib for 27 CCLE liver cancer cell lines, according to *MUC16* mutational status. The results revealed that cells harboring in MMs in *MUC16* exhibited a tendency of higher sensitivity to regorafenib than those with no or single *MUC16* mutations, pointing to the potential value of MMs as a predictive marker for targeted therapies.

Clinical outcomes and MMs in MUC16

To assess the clinical impact of MMs in individual oncogenes, the clinicopathological factors according to mutational status in *MUC16* were investigated (**Table 2)**. MMs in *MUC16* was associated with viral hepatitis, higher tumor markers and vascular invasion. Patient RFS was significantly worse in the group with *MUC16* MMs than in the group with *MUC16* SM ($P = 0.022$), although there was no significant difference between the group with *MUC16* SM and the group with wild-type *MUC16* ($P = 0.324$, **Fig. 3a**). Using TCGA data sets, we checked HCC-specific survival according to mutational status in *MUC16* (**Fig. 3b**). No significant differences in Kaplan-Meier survival curves were observed between the group with *MUC16* SM and the group with wild-type *MUC16* ($P = 0.616$). Patient HCC-specific survival was significantly worse in the group with *MUC16* MMs than in the group with *MUC16* SM ($P = 0.043$) and in the group with wild-type *MUC16* ($P = 0.013$).

Discussion

The results revealed that MMs accumulated selectively in specific genes and a high frequency of mutations in genes did not always correspond to a high frequency of MMs. The signature of mutations identified as MMs, including mutation type and functional impact, also varied compared with that of mutations identified as SM. Furthermore, the GEP results implied that MMs had a greater impact on gene expression than SMs in some oncogenes, a trend was identified that MMs led to additional up-regulation (gain-of-function) of gene expression. Consequently, MMs are not just a reflection of mutation burden but occur in specific genes and pathways and thus contribute to carcinogenesis and/or acquisition of malignant potential in HCC.

The overall landscape of MMs was recently reported through a pan-cancer analysis of 60,954 cancer samples [5]. The study identified that oncogenic MMs were a relatively common driver event and thus MMs provide a novel underlying mechanism for cancer development. These observations reinforce the idea that MMs in the same oncogene cooperate to potentiate tumor-promoting activity. These findings also indicate the potential usefulness of MMs as a biomarker and a target for molecular-targeted therapy. However, the signature and clinical relevance of MMs in HCCs had remained unclear. In the present study, detailed clinical information led to the validation of the clinical significance of MMs in HCCs, and the present results identified MMs as an independent predictor for

prognosis in HCC. To date, there has been no report on the clinical relevance of MMs, and therefore the present study provides further evidence of the prognostic impact of MMs.

The clinical impact of MMs in some genes in a sample led us to assess the clinical impact of MMs in individual oncogenes. Aberrant activation of WNT/β-catenin signaling is a driving molecular event in a wide range of tumors, including HCCs [19]. Somatic missense mutations in exon 3 of *CTNNB1* are frequently reported in HCCs (10.0–32.8% in genome-wide sequencing studies) [3] [20]. Consistent with previous reports, we found that mutations in *CTNNB1* were frequently identified in HCC and MMs in *CTNNB1* were also frequently found in 14% (11/79) of samples with at least one mutation. However, a significant prognostic difference between SM and MMs in *CTNNB1* was not identified (data not shown). The conflicting prognostic impact of mutated *CTNNB1* due to the bilateral nature was reported. HCC cases with the existence of an interaction between WNT activation and TGF-beta activation show poor survival, whereas HCCs harboring mutant *CTNNB1* show generally favorable prognosis [21]. Therefore, for further investigation, we focused on *MUC16*, in which MMs were identified in 15.2% (7/46) of mutated samples, as a candidate oncogene to validate the impact of MMs.

MUC16, which encodes a protein also known as ovarian carcinoma antigen CA125, has been recognized as a crucial factor in hepatocarcinogenesis [22] and its usefulness as a diagnostic and prognostic marker has been demonstrated [23]. The present results demonstrated that the presence of MMs in *MUC16* was associated with viral hepatitis, higher tumor markers and vascular invasion. Patient RFS was significantly worse in the group with MMs than in the group with SM, although there was no significant difference between the group with SM and the group with wild-type *MUC16.* The findings support the idea that MMs in the same oncogene cooperate to potentiate tumor-promoting activity.

The findings that the accumulation of MMs in specific oncogenes led us to assess the impact of MMs on phenotypes in cancer cell lines. Analysis of drug sensitivity screens in CCLE cell lines [18] revealed that cells harboring in MMs in *MUC16* exhibited a higher sensitivity to regorafenib than those with no or single *MUC16* mutations, indicating the potential value of MMs as a predictive marker for targeted therapies. Similarly, a previous study [5] reported that cells harboring MMs in *PIK3CA* exhibited a higher sensitivity to PI3K inhibitors compared with those with no or single *PIK3CA* mutations, suggesting that MMs may be useful as predictive markers for targeted therapies. The correlation between mutational status in an oncogene and the sensitivity to the targeted drug for the molecule should be further explored in future studies.

During recent years, new immune-modulatory agents have been introduced for HCC treatment, eventually leading to the clinical breakthrough of immune checkpoint inhibitors (ICIs) targeting programmed death-1 (PD-1), programmed death-ligand 1 (PD-L1), or cytotoxic T lymphocyte antigen-4 (CTLA-4) [24]. The TMB, which correlates with MMs, has received increasing attention owing to its potential to estimate the efficacy of the response to ICI. The present results indicated that MMs were associated with the TMB to some extent; however, MMs were not just a reflection of mutation burden but occurred in specific genes and pathways through a selection mechanism to contribute to carcinogenesis and/or acquisition of malignant potential. These findings suggest the potential utility of MMs as a biomarker for ICIs. Future studies should examine the usefulness of MMs as a biomarker for ICIs.

This study had several limitations. First, the number of MMs in each specific gene was relatively small, and therefore investigation of the clinical significance of MMs in each gene could not be conducted because of under power statistics. Ideally, the clinical significance of MMs in specific candidate genes will be investigated to

establish a biomarker for targeted therapy. Second, the previous study [5] reported that the proportion of MMs in cis was particularly high (86%) in oncogenes with MMs. Consistent with the previous reports, most MMs (83%) in *CTNNB1* were present in cis in the present study. However, all of the MMs in *MUC16* was not located in a same amplicon, therefore the allelic configuration of MMs in *MUC16* could not be investigated.

In conclusions, MMs are a relatively common driver event that occur selectively in specific genes and have a role in tumor-promoting activity. To the best of our knowledge, this is the first study investigating the clinical significance of MMs in patients with HCC. The present findings provide important insights into the development of personalized screening and management strategies for HCCs.

Declarations

Funding

Not applicable

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Ethics approval

We used archived tissue samples for this study that were obtained with patient consent at Shizuoka Cancer Center. The study was approved by the ethics committee of Shizuoka Cancer Center. The study protocol conforms to the ethics guidelines of the 1975 Declaration of Helsinki.

Consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

Data for this study is confidential patient information regulated by the IRB of the institution. Requests to access data will have to be in compliance with the institutional IRB.

Code availability

Not applicable

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Taisuke Imamura, Yukiyasu Okamura and Keiichi Ohshima. The first draft of the manuscript was written by Taisuke Imamura and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

References

1. Bray F, Ferlay J, Soerjomataram I, et al. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018;68:394–424.
2. Neuveut C, Wei Y, Buendia MA. Mechanisms of HBV-related hepatocarcinogenesis. J Hepatol. 2010;52:594–604.
3. Shibata T, Aburatani H. Exploration of liver cancer genomes. Nat Rev Gastroenterol Hepatol. 2014;11:340–9.
4. Nault JC, Mallet M, Pilati C, et al. High frequency of telomerase reverse-transcriptase promoter somatic mutations in hepatocellular carcinoma and preneoplastic lesions. Nat Commun. 2013;4:2218.
5. Saito Y, Koya J, Araki M, et al. Landscape and function of multiple mutations within individual oncogenes. Nature. 2020;582:95–9.
6. Garraway LA, Lander ES. Lessons from the cancer genome. Cell. 2013;153:17–37.
7. Vogelstein B, Papadopoulos N, Velculescu VE, et al. Cancer genome landscapes Science. 2013;339:1546–58.
8. Yamaguchi K, Urakami K, Ohshima K, et al. Implementation of individualized medicine for cancer patients by multiomics-based analyses-the Project HOPE. Biomed Res. 2014;35:407–12.
9. Liu J, Lichtenberg T, Hoadley KA, et al. An Integrated TCGA Pan-Cancer Clinical Data Resource to Drive High-Quality Survival Outcome Analytics. Cell. 2018;173:400–16 e411.
10. Hatakeyama K, Ohshima K, Nagashima T, et al. Molecular profiling and sequential somatic mutation shift in hypermutator tumours harbouring POLE mutations. Sci Rep. 2018;8:8700.
11. Nagashima T, Shimoda Y, Tanabe T, et al. Optimizing an ion semiconductor sequencing data analysis method to identify somatic mutations in the genomes of cancer cells in clinical tissue samples. Biomed Res. 2016;37:359–66.
12. Ohshima K, Hatakeyama K, Nagashima T, et al. Integrated analysis of gene expression and copy number identified potential cancer driver genes with amplification-dependent overexpression in 1,454 solid tumors. Sci Rep. 2017;7:641.
13. Sondka Z, Bamford S, Cole CG, et al. The COSMIC Cancer Gene Census: describing genetic dysfunction across all human cancers. Nat Rev Cancer. 2018;18:696–705.
14. Chakravarty D, Gao J, Phillips SM, et al. OncoKB: A Precision Oncology Knowledge Base. JCO Precis Oncol 2017; 2017.
15. Nagashima T, Yamaguchi K, Urakami K, et al. Japanese version of The Cancer Genome Atlas, JCGA, established using fresh frozen tumors obtained from 5143 cancer patients. Cancer Sci. 2020;111:687–99.
16. Rosenthal R, McGranahan N, Herrero J, et al. DeconstructSigs: delineating mutational processes in single tumors distinguishes DNA repair deficiencies and patterns of carcinoma evolution. Genome Biol. 2016;17:31.
17. Letouzé E, Shinde J, Renault V, et al. Mutational signatures reveal the dynamic interplay of risk factors and cellular processes during liver tumorigenesis. Nature Communications 2017; 8.
18. Ghandi M, Huang FW, Jané-Valbuena J, et al. Next-generation characterization of the Cancer Cell Line Encyclopedia. Nature. 2019;569:503–8.
19. Polakis P. Wnt signaling and cancer. Genes Dev. 2000;14:1837–51.
20. de La Coste A, Romagnolo B, Billuart P, et al. Somatic mutations of the beta-catenin gene are frequent in mouse and human hepatocellular carcinomas. Proc Natl Acad Sci U S A. 1998;95:8847–51.

21. Hoshida Y, Nijman SM, Kobayashi M, et al. Integrative transcriptome analysis reveals common molecular subclasses of human hepatocellular carcinoma. Cancer Res. 2009;69:7385–92.
22. Kasprzak A, Adamek A. Mucins: the Old, the New and the Promising Factors in Hepatobiliary Carcinogenesis. Int J Mol Sci 2019; 20.
23. Zhou S, Wang Z, Li M, Wu L. Elevated Preoperative Serum CA125 Predicts Larger Tumor Diameter in Patients with Hepatocellular Carcinoma and Low AFP Levels. Biomed Res Int 2019; 2019: 6959637.
24. Herbst RS, Baas P, Kim DW, et al. Pembrolizumab versus docetaxel for previously treated, PD-L1-positive, advanced non-small-cell lung cancer (KEYNOTE-010): a randomised controlled trial. Lancet. 2016;387:1540–50.

Tables

Table 1 Univariate and multivariate analyses for relapse-free survival after hepatectomy

Variable			Univariate	Multivariate		
			P-value [a]	HR	95% CI	*P*-value [b]
Genetic signatures						
	Tumor mutation burden	Hyper	0.195			
		Hypo				
	Multiple mutations	Present	**0.012**	1.72	1.01-3.17	**0.045**
		Absent				
Clinicopathological factors						
	Sex	Male	0.402			
		Female				
	Age, years	≥ 70	0.893			
		< 70				
	HBV or HCV	Negative	0.655			
		Positive				
	ICG-R15, %	≥ 20	0.424			
		< 20				
	AFP, ng/ml	≥ 200	0.106			
		< 200				
	PIVKA-II, mAU/ml	≥ 100	**0.001**	1.34	0.88–2.06	0.177
		< 100				
	Tumor size, mm	≥ 30	**0.006**	1.23	0.84–1.96	0.257
		< 30				
	Macrovascular invasion	Positive	**0.004**	1.72	0.86–3.10	0.119
		Negative				

	Microvascular invasion	Positive	**< 0.001**	1.96	1.36–2.83	**< 0.001**
		Negative				
	Liver damage	Liver cirrhosis	0.085			
		No cirrhosis				

[a]Kaplan–Meier method. Significance was determined by the log-rank test. [b]Multivariate survival analysis was performed using Cox's proportional hazard model. HR: hazard ratio; CI: confidence interval; HBV, hepatitis B virus; HCV, hepatitis C virus; ICG, indocyanine green; RFS, relapse-free survival; AFP, alpha fetoprotein. Significant values are in bold.

Table 2 Clinicopathological factors according to the number of *MUC16* mutations

		Wild-type	Single mutation	Multiple mutations	P-value		
Variable		N=176	N=38	N=7	Wild vs. Multiple	Single vs. Multiple	Wild vs. Single
Gender, N, (%)	Male	144 (82%)	30 (79%)	6 (86%)	0.787	0.671	0.684
	Female	32 (18%)	8 (21%)	1 (14%)			
Age, years, (IQR)		70 (64–76)	74 (68–78)	65 (64–73)	0.196	**0.014**	0.030
HBV or HCV, N, (%)	HCV	53 (30%)	11 (29%)	5 (71%)	**0.047**	0.060	0.974
	HBV	34 (19%)	7 (18%)	0 (0%)			
	NBNC	89 (51%)	20 (53%)	2 (29%)			
ICG-R15, %, (IQR)		9.5 (6.1–13.7)	10.7 (7.5–15.7)	13.4 (5.9–15.1)	0.313	0.719	0.136
AFP, ng/ml, (IQR)		9.2 (3.7–144.1)	5.2 (2.3–24.2)	641.4 (7.4–883.7)	0.150	**0.043**	**0.045**
PIVKAII, mAU/ml, (IQR)		116.0 (32.6–1495.0)	414.0 (24.8–3537.5)	5670.0 (160.0–37800.0)	**0.048**	0.183	0.454
Tumor size, mm, (IQR)		35 (22–65)	36 (25–76)	63 (33–78)	0.392	0.730	0.335
Macrovascular invasion, N, (%)	Positive	11 (6%)	1 (3%)	2 (29%)	**0.024**	**0.036**	0.338
Microvascular invasion, N, (%)	Positive	57 (32%)	14 (37%)	2 (29%)	0.831	0.670	0.599

continuous variables expressed as median and interquartile range (IQR). HBV, hepatitis B virus; HCV, hepatitis C virus; ICG, indocyanine green; AFP, alpha fetoprotein; NBNC, non B non C. Significant values are in bold.

Figures

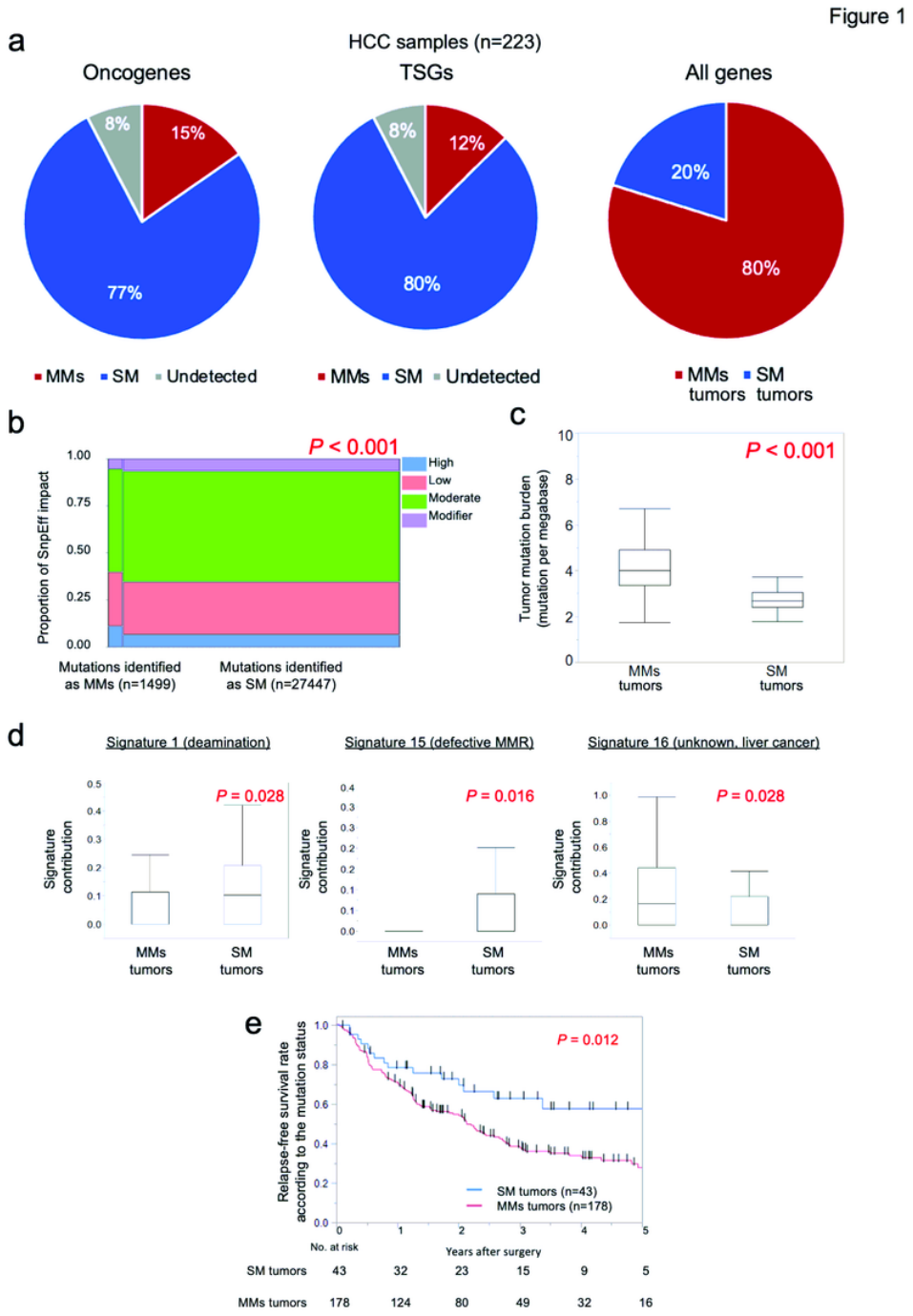

Figure 1

Fraction of multiple mutations in individual genes. (a) Percentages of HCCs with single mutation (SM) and multiple mutations (MMs) according to the classification for oncogenes and tumor suppressor genes (TSGs). (b) Comparison of the impact of genomic variant annotations and functional effect by SnpEff. (c) A comparison of TMB between MMs tumors and SM tumors. The TMB was significantly higher in MMs tumors than that in SM tumors. (d) Comparisons of signature contributions between MMs tumors and SM tumors. Three signature scores were significantly varied betweem MMs tumors and SM tumors. (e) Prognostic analysis according to the

presence of MMs. The RFS was significantly worse in the group with MMs tumors than in the group with SM tumors (P = 0.012, log-rank test).

Figure 2

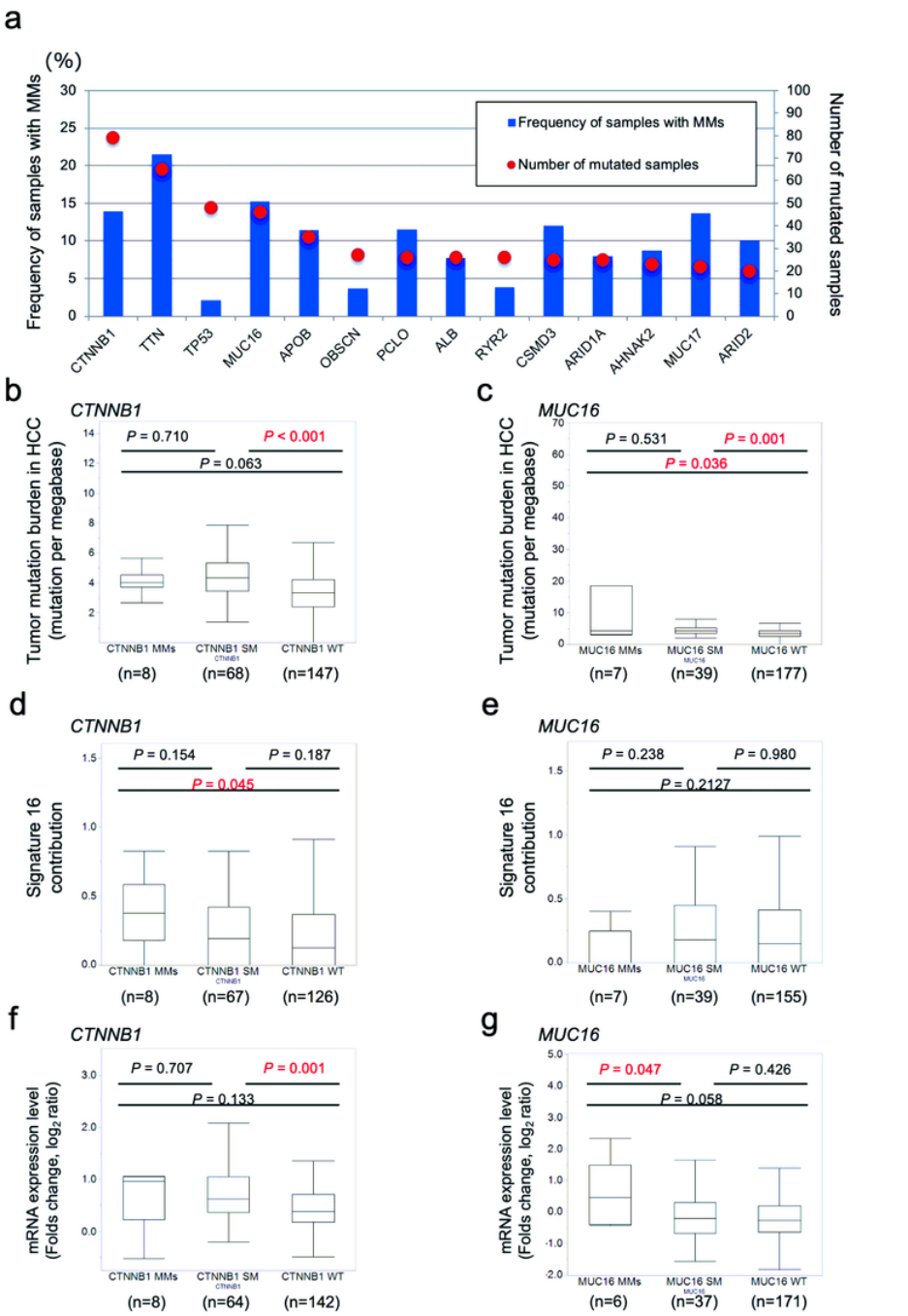

Figure 2

Frequent MMs in a variety of oncogenes in HCC. (a) The number of mutated samples and the fraction of samples with MMs for 14 genes with 20 or more mutated samples in the present cohort (n = 223). Correlations between MMs in CTNNB1 (b) and MUC16 (c) and TMB were found between samples with SM and samples with the wild-type gene (CTNNB1, P < 0.001; MUC16, P = 0.001, Mann–Whitney U test) but not between SM and MMs (CTNNB1, P = 0.710, MUC16, P = 0.531, Mann–Whitney U test). The signature 16 was significantly higher in samples with MMs in CTNNB1 than samples with the wild-type CTNNB1, although no significant differences in

the signature score between samples with SM in CTNNB1 and samples with the wild-type CTNNB1 (d). No significant differences in the signature 16 score according tot the mutational status in MUC16 (e). The impact of MMs on gene expression in CTNNB1 (f) and MUC16 (g).

Figure 3

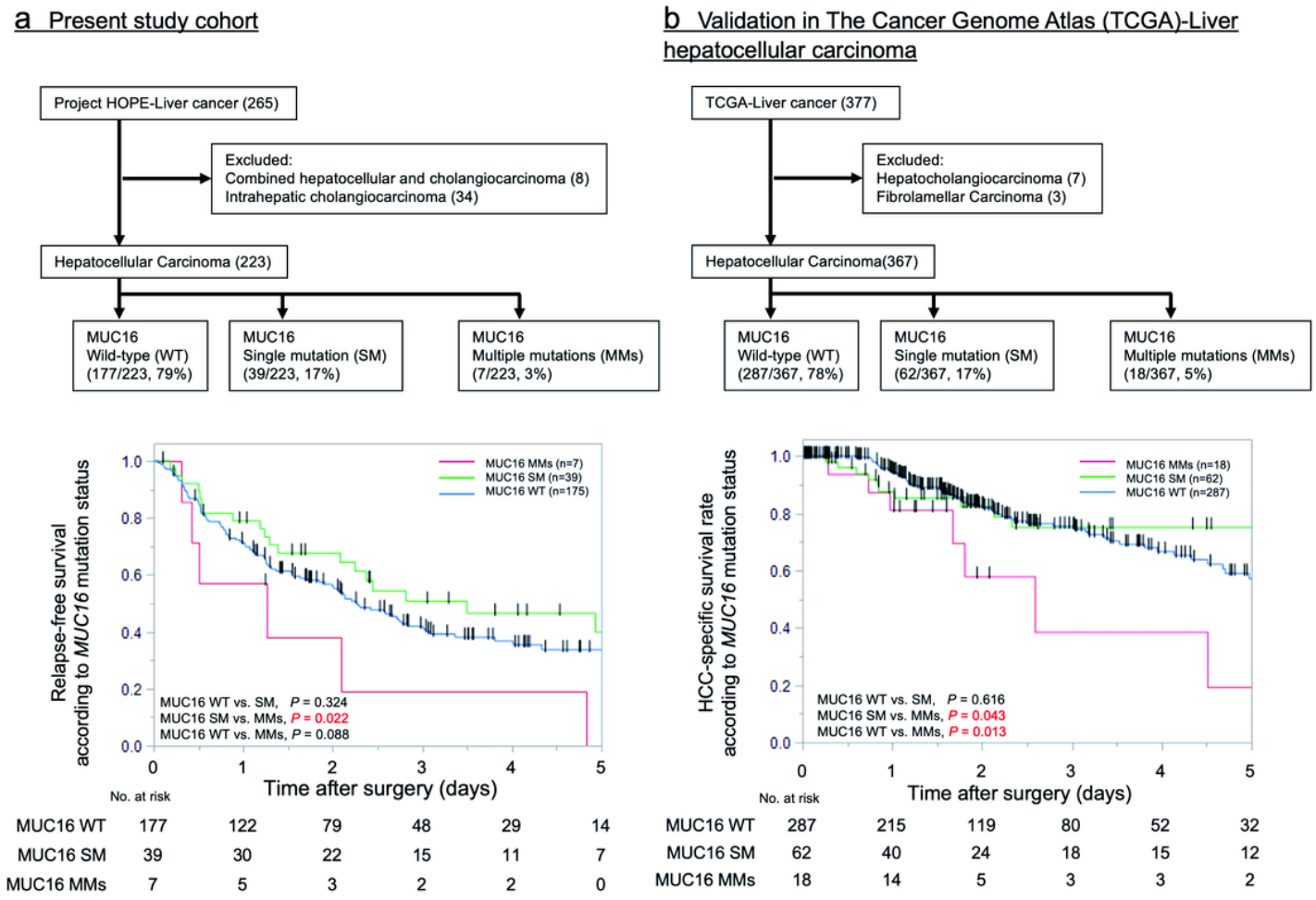

Figure 3

Prognostic impact of MMs in MUC16 and its validation in TCGA. The frequency of SM and MMs in MUC16 was 17% and 3% in our study and 17% and 5% in TCGA. (a) Patient RFS was significantly worse in the group with MUC16 MMs than in the group with MUC16 SM (P = 0.022), although there was no significant difference between the group with MUC16 SM and the group with wild-type MUC16 in our cohort (P = 0.324, log-rank test). Patient HCC-specific survival was significantly worse in the group with MUC16 MMs than in the group with MUC16 SM in TCGA data set (P = 0.043, log-rank test).

Supplementary Files

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