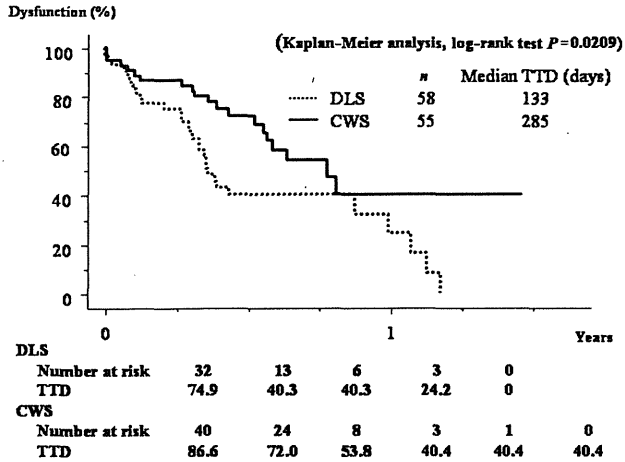

Fig. 5. Time to dysfunction (TTD) using the Kaplan–Meier method and log–rank test. The Covered Wallstent (CWS) had a significantly longer TTD than the DoubleLayer stent (DLS) ($P = 0.0209$).

has a longer reported patency than other types of PS.[15] However, the CWS was patent significantly longer than the DLS in this study.

This study enrolled only patients with PHC, whereas many reports on stenting for malignant biliary obstruction included various causative diseases. In a randomized study comparing covered and uncovered Diamond stents by the first author, differences were noted in the effectiveness of CMS according to the causative disease.[9] For PHC, the CMS was patent significantly longer than the UMS. Therefore, we think that the CMS is the first-line stent to choose for PHC with obstructive jaundice.

The removal of the CWS succeeded without any complications in all cases where it was required, although this stent has uncovered portions in both ends. A fully covered MS could be removed more easily and should be used for benign cases or resectable cases as a bridge to surgery.[3,20,21] For pancreatic cancer, new chemotherapeutic agents, such as gemcitabine, have prolonged survival times. Therefore, an exchangeable CMS may be suitable for the ongoing management of PHC.

The incidence of complications other than stent occlusion was higher in the CWS group. The prognosis of cholecystitis after CMS placement is affected by tumor involvement of the orifice of the cystic duct (OCD) and gallbladder stones.[22,23] In our study, of the four cholecystitis cases, OCD involvement was observed in one case, gallbladder stones in one case, and no data on the status of the OCD or gallbladder stones were available in two cases. Only one case developed pancreatitis in our series. Prior early stent thrombosis may reduce the incidence of pancreatitis compared with previous studies using the CMS.[4] Another problem with the CWS was the high migration rate (9.1%). The first author reported a lower incidence of migration with the ComVi stent, which has an outer uncovered layer as an anchor.[24] An anchoring system is needed for the CMS to prevent migration.

Migration is a serious complication that reduces the patient's quality of life. Migration was not recognized as stent occlusion, and was not reflected in the calculation of stent patency in many articles. We tried to estimate the TTD using Kaplan–Meier analysis. We think that this new method may surrogate real stent function.

The median patency of the DLS in this study (133 days) was higher than that of a conventional straight type PS (90–120 days). The previously reported median patency of the DLS (144 days) was similar to our result. We think that the DLS is superior to other types of PS based on our study, especially in PHC. Therefore, we consider that DLS should be selected for the patients with poor condition and expected prognosis of less than 3 months. The Tannenbaum plastic stent was reported to have a higher cost-effectiveness than the uncovered metallic stent.[25] In this study, the cost-effectiveness analysis was not performed, and we should clarify this issue in the near future. The disadvantage of the DLS was the bile duct kinking at the proximal stent end due to its stiffness. Two DLS were occluded because of bile duct kinking despite careful insertion to avoid this complication.

In conclusion, the CWS was patent significantly longer and had a longer TTD than the DLS in the management of PHC with obstructive jaundice. The incidence of complications other than stent occlusion was higher with the CWS, but the difference did not reach significance. Improvement in the design of the CWS is needed to prevent these complications.

ACKNOWLEDGMENTS

We wish to thank the following investigators.
Former and current Chairman of Endoscopic Forum Japan (EFJ):

- Masatsugu Nakajima, Department of Gastroenterology, Kyoto Second Red Cross Hospital
- Hisao Tajiri, Department of Endoscopy, The Jikei University School of Medicine.

Other participants of this study:

- Yousuke Nakai, Naoki Sasahira, Kenji Hirano, Natsuyo Yamamoto, Saburou Matsubara, Osamu Togawa, Toshihiko Arizumi, Yukiko Ito, Hirofumi Kogure, Takashi Sasaki and Masao Omata, Department of Gastroenterology, Graduate School of Medicine, The University of Tokyo
- Tsuyoshi Mukai, Department of Gastroenterology, Gifu Municipal Hospital
- Hirotoshi Iwano and Noriko Ishigaki, Department of Gastroenterology & Hepatology, Yamaguchi University Graduate School of Medicine
- Yasuhide Ochi, Department of Gastroenterology, Nagano Municipal Hospital
- Tadayuki Takagi, Tsunehiko Ikeda, Rei Suzuki and Hiromasa Ohira, Department of Gastroenterology and Rheumatology, Fukushima Medical University School of Medicine
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Research

Whole-exome sequencing of human pancreatic cancers and characterization of genomic instability caused by *MLH1* haploinsufficiency and complete deficiency

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Whole-exome sequencing (Exome-seq) has been successfully applied in several recent studies. We here sequenced the exomes of 15 pancreatic tumor cell lines and their matched normal samples. We captured 162,073 exons of 16,954 genes and sequenced the targeted regions to a mean coverage of 56-fold. This study identified a total of 1517 somatic mutations and validated 934 mutations by transcriptome sequencing. We detected recurrent mutations in 56 genes. Among them, 41 have not been described. The mutation rates varied widely among cell lines. The diversity of the mutation rates was significantly correlated with the distinct *MLH1* copy-number status. Exome-seq revealed intensive genomic instability in a cell line with *MLH1* homozygous deletion, indicated by a dramatically elevated rate of somatic substitutions, small insertions/deletions (indels), as well as indels in microsatellites. Notably, we found that *MLH1* expression was decreased by nearly half in cell lines with an allelic loss of *MLH1*. While these cell lines were negative in conventional microsatellite instability assay, they showed a 10.5-fold increase in the rate of somatic indels, e.g., truncating indels in *TP53* and *TGFBR2*, indicating *MLH1* haploinsufficiency in the correction of DNA indel errors. We further analyzed the exomes of 15 renal cell carcinomas and confirmed *MLH1* haploinsufficiency. We observed a much higher rate of indel mutations in the affected cases and identified recurrent truncating indels in several cancer genes such as *VHL*, *PBRM1*, and *JARID1C*. Together, our data suggest that *MLH1* hemizygous deletion, through increasing the rate of indel mutations, could drive the development and progression of sporadic cancers.

[Supplemental material is available for this article.]

The current understanding of cancer is that it arises as a result of the accumulation of genetic and epigenetic mutations that confer a selective advantage to the cells in which they occur (Vogelstein and Kinzler 2004; Greenman et al. 2007; Stratton et al. 2009). Over the past quarter of a century, many efforts have been made to learn about the causative mutations that drive various types of cancer, including pancreatic cancer, one of the most lethal forms of human cancer. By using the Sanger sequencing method, i.e., PCR amplification followed by plasmid subcloning and DNA sequencing, previous studies have identified thousands of genetic alterations in the cancer genome and provided important insights into the pancreatic cancer biology (Jones et al. 2008; Maitra and Hruban 2008). However, because Sanger sequencing is performed on single amplicons, its throughput is limited, and large-scale sequencing projects are expensive and laborious (Schuster 2008; Metzker 2010). Moreover, it has been reported that it has a limited sensitivity to recognize the mutant DNA allele if it is present in a minor population of cancer cells (Nakahori et al. 1995; Thomas et al. 2006; Qiu et al. 2008). In addition, the bacterial cloning workflows tend to be complex and time-consuming, and bias can be introduced into this step (Thomas et al. 2006).

The advent of next-generation sequencing (NGS) technologies has brought a high level of efficiency to genome sequencing (Schuster 2008; Metzker 2010). The enriched DNA is sequenced directly, avoiding the cloning step (Ng et al. 2009). While whole-genome

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E-mail haburata-tky@umin.ac.jp.
Article published online before print. Article, supplemental material, and publication date are at http://www.genome.org/cgi/doi/10.1101/gr.123109.111.

22:208–219 © 2012 by Cold Spring Harbor Laboratory Press; ISSN 1088-9051/12; www.genome.org

sequencing is the most complete, it remains sufficiently expensive that cost-effective alternatives are important. Target-enrichment strategies allow the selective capture of the genomic regions of interest. Whole-exome sequencing (Exome-seq) through integrating two systems has enabled us to concentrate our sequencing efforts on the protein-coding exons in the human genome. This approach is substantially cost- and labor-efficient (Schuster 2008; Metzker 2010; Biesecker et al. 2011). Moreover, by taking advantage of deep coverage of target regions, it shows an excellent sensitivity for the detection of variants with a minor allele frequency down to 2% (Li et al. 2010). Recent studies have successfully applied Exome-seq to identify genetic changes involved in Mendelian diseases (Choi et al. 2009; Ng et al. 2010). In addition to Exome-seq, full-length transcriptome sequencing (mRNA-seq) offers a fast and inexpensive alternative. It is an easier method to identify coding sequences and capture variants in genes that are expressed, as well as to generate additional information, such as gene expression level and splicing patterns (Sugarbaker et al. 2008; Cirulli et al. 2010).

Genomic instability is a characteristic feature of almost all human cancers (Lengauer et al. 1998; Negrini et al. 2010). Its molecular basis is well understood in hereditary cancers, in which it has been linked to mutations in DNA mismatch repair (MMR) genes. One of the best-documented examples is the hereditary nonpolyposis colon cancer (HNPCC). In general, MMR defects are the result of a germline mutation in one of the MMR genes followed by a hit on the second allele of that gene, or methylation of the promoter of a MMR gene, usually *MLH1*, resulting in the loss of protein function (Fishel et al. 1993; Hemminki et al. 1994). In contrast, the molecular basis of genomic instability in sporadic cancers remains unclear (Negrini et al. 2010).

In the past few years, by use of Sanger sequencing, several consortia have scanned the coding sequences of 18,191–20,661 genes in carcinomas of the colon, breast, and pancreas and in glioblastomas (Sjoblom et al. 2006; Wood et al. 2007; Jones et al. 2008; Parsons et al. 2008). These genome-wide studies reported that mutations targeting caretaker genes (DNA repair genes and mitotic checkpoint genes) were infrequent. To date, no statistical correlation has been described in sporadic cancers between the allelic loss of a caretaker gene and the increased rate of genomic instability. It has been thought that a single copy of the wild-type allele of a caretaker gene is sufficient to perform its normal function, and both alleles of the gene would have to be inactivated before the genome becomes unstable (Bodmer et al. 2008; Negrini et al. 2010). Since the occurrence of two independent somatic mutations at both alleles of the same gene is likely to represent a very rare event (Bodmer et al. 2008), these studies argued that mutations in caretaker genes probably do not account for the presence of genomic instability in many sporadic cancers (Negrini et al. 2010).

We here performed Exome-seq on 15 pancreatic ductal adenocarcinoma (PDAC)–derived cell lines. This study identified 1517 somatic mutations and validated 934 of them by mRNA-seq. We notably found a significant correlation between *MLH1* allelic loss and the increased rate of somatic indel mutations, and we further confirmed this finding in primary renal cell carcinomas (RCCs). In the affected cases, we detected recurrent truncating indels that inactivate tumor suppressor genes, such as *TP53*, *TGFBR2*, and *VHL*. We also observed a higher prevalence of indels in the coding microsatellite sequences. Our data, therefore, indicate that deletion of one copy of the *MLH1* gene results in haploinsufficiency in the correction of DNA indel errors and could be a driving force in pancreatic and renal carcinogenesis.

Results

The performance of Exome-seq

We sequenced the exomes of 15 PDAC-derived cell lines and their matched normal samples (Table 1). On average, 6.6 Gb of high-quality sequence data (about 44.2 million paired 75-base reads) were generated per sample. More than 88% of the sequence reads were uniquely aligned to the human reference genome with the expected insert size and correct orientations, and 68.4% of them fell within the targeted regions (Fig. 1A; Supplemental Fig. S1). The average fold-coverage of each exome was 56× (Supplemental Fig. S2). On average per exome, 96.9% of targeted bases were covered by at least one read, and 83.4% of targeted bases were covered by at least 10 reads (Fig. 1B; Supplemental Fig. S3).

An overview of somatic mutations

By using Exome-seq, we identified a total of 1517 somatic mutations, including 39 nonsense, 833 missense, 423 synonymous substitutions, and 49 substitutions in untranslated regions (UTRs), 137 frame-shift indels and 36 in-frame indels (Fig. 1C). The complete list

Table 1. Characteristics of pancreatic tumor cell lines

Sample OID	Carcinoma type	Pathology	Differentiation	Lymph node metastasis	Tissue derivation	Sample type	*MLH1* status
PA018	Ductal adenocarcinoma	Tubular	Moderately	–	Primary pancreatic tumor	Cell line	LOH
PA028	Ductal adenocarcinoma	Tubular	Moderately	+	Primary pancreatic tumor	Cell line	ROH
PA055	Ductal adenocarcinoma	Tubular	Moderately	+	Primary pancreatic tumor	Cell line	LOH
PA086	Ductal adenocarcinoma	Tubular	Moderately	+	Primary pancreatic tumor	Cell line	ROH
PA090	Ductal adenocarcinoma	Tubular	Well	+	Primary pancreatic tumor	Cell line	ROH
PA107	Ductal adenocarcinoma	Invasive	Moderately to well	–	Primary pancreatic tumor	Cell line	ROH
PA122	Ductal adenocarcinoma	Invasive	Moderately to poorly	–	Primary pancreatic tumor	Cell line	ROH
PA167	Ductal adenocarcinoma	Invasive	Moderately	+	Primary pancreatic tumor	Cell line	LOH
PA182	Ductal adenocarcinoma	Invasive	Moderately	+	Primary pancreatic tumor	Cell line	ROH
PA195	Ductal adenocarcinoma	Tubular	Moderately	+	Primary pancreatic tumor	Cell line	ROH
PA202	Ductal adenocarcinoma	Tubular	Moderately	+	Primary pancreatic tumor	Cell line	LOH
PA215	Ductal adenocarcinoma	Tubular	Poorly	+	Primary pancreatic tumor	Cell line	ROH
PA254	Ductal adenocarcinoma	Tubular	Moderately	–	Primary pancreatic tumor	Cell line	ROH
PA285	Ductal adenocarcinoma	Invasive	Moderately	–	Primary pancreatic tumor	Cell line	HD
PA333	Ductal adenocarcinoma	Tubular	Well	+	Primary pancreatic tumor	Cell line	ROH

ROH indicates retention of heterozygosity; LOH, loss of heterozygosity; and HD, homozygous deletion.

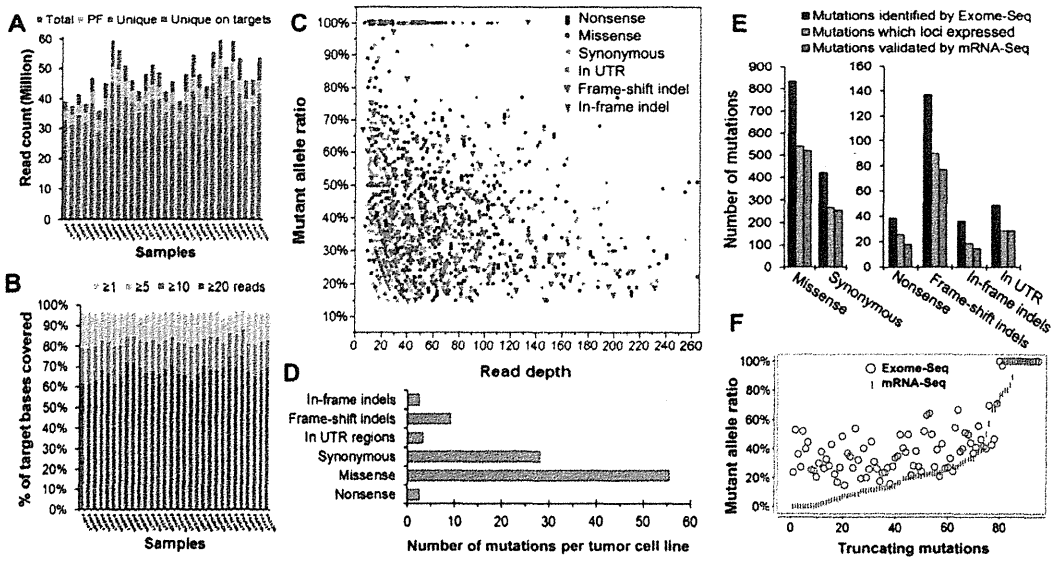

Figure 1. The performance of Exome-seq and a summary of somatic mutations. (*A*) The summary of Exome-seq data. For each sample, the number of raw sequence reads (total), passing filter reads (PF), unique reads that mapped in consistent read pairs (unique), and the unique reads that fall within the targeted regions (unique on target) are shown. (*B*) The sequence coverage of targeted bases. The fraction of the targeted bases that were covered by unique reads at the sequence depth of 1×, 5×, 10×, and 20× is shown. (*C*) An overview of the somatic mutations identified by Exome-seq. Different markers and colors were used to show different mutation types. (*D*) The average number of somatic mutations identified per tumor cell line. (*E*) The performance of mRNA-seq in verification of somatic mutations identified by Exome-seq. The mutations that loci expressed represent those mutations that loci covered by five or more cDNA sequence reads. (*F*) Validation of the truncating mutations that introduced premature termination codons. The abundance of the mutant alleles in genomic DNA was compared with that of their corresponding cDNA.

of 1517 somatic mutations is shown in Supplemental Table S1. On average, each cell line contains 101 somatic mutations, 89% of which are base substitutions (Fig. 1D). The frequencies of mutant alleles ranged from 15%–100%, with a median of 41%. The depth of coverage at the mutation loci ranged from 10× to 637×, with a median of 42× (Fig. 1C; Supplemental Table S1). The lengths of somatic small indels varied from 1–29 bp. Seventy-eight percent of the indels were 1–3 bp in length (Supplemental Fig. S4). By using genome-wide SNP array, we identified more than 50 focal homozygous deletions (Supplemental Table S2). The *CDKN2A* locus at *9p21.3* and the *SMAD4* locus at *18q21.2* were frequently deleted in the tumor cell lines analyzed (Supplemental Fig. S5). The somatic mutations mainly clustered in nine signaling pathways, as shown in Supplemental Figure S6A. The background mutation rate estimated for targeted exonic regions was 2.7 mutations per megabase of DNA sequences.

Validation of somatic mutations using mRNA-seq

In total, 61.6% (934 out of 1517) of the mutations identified by Exome-seq were validated by mRNA-seq. If we focus on the expressed genes, 94.3% (914 out of 969) of the mutations at those loci covered by five or more cDNA sequence reads were successfully validated by mRNA-seq (Fig. 1E). Additionally, 20 mutations at the loci with a lower coverage (less than five reads, but three reads or more) were also confirmed by mRNA-seq. The percentages of mutations validated by mRNA-seq varied across mutation types. Generally, the validation ratio of truncating mutations is lower than that of nontruncating mutations.

For truncating mutations (Fig. 1F), the abundance of the mutant allele in the cDNA appears to be relatively lower than that of their corresponding genomic DNA (gDNA). Despite the lower abundance, mRNA-seq was still able to confirm 81 of those 94 (86.2%) truncating mutations at loci covered by five or more cDNA sequence reads. The remaining 13 truncating mutations were all heterozygous. Their loci were covered moderately well, but no mutant alleles were observed in the cDNA sequences. We performed Sanger sequencing to confirm if they resulted from the false-positive events of Exome-seq. As shown in Supplemental Figure S7, 12 of the 13 truncating mutations were successfully validated by Sanger sequencing. The mutant alleles were only detected in the gDNA of the tumor cell lines rather than in their cDNA, suggesting the transcripts carrying the mutant alleles were probably degraded through the nonsense-mediated mRNA decay (NMD) pathway (Holbrook et al. 2004). One mutation was found to be false-positive, possibly caused by mapping errors.

The recurrently mutated genes

In this study, 1359 genes were identified with somatic mutations. Among them, 56 genes were recurrently mutated in two or more cell lines (Table 2). The mutation rate of these genes was much higher than the background level. The most frequently mutated gene was *KRAS*, followed by *CDKN2A*, *TP53*, and *SMAD4*. Mutation of these four genes and 11 other genes has been reported either in the COSMIC database (http://www.sanger.ac.uk/genetics/CGP/cosmic/) or in a previous study (Jones et al. 2008), as shown in Supplemental Figure S8, while mutation of the remaining 41 genes, to our knowledge, has not been described in PDAC. Totally, 150 point mutations were identified in the 56 recurrently mutated genes. Among them, 109 mutations in 40 genes were confirmed by mRNA-seq (Supplemental Table S1). For the remaining 41 mutations that were not confirmed by mRNA-seq, seven loci were poorly expressed (covered by two or fewer cDNA sequence reads) and 34 loci were not expressed at all.

Table 2. The recurrently mutated genes

Rank	#Gene symbol	Coding sequence length (bases)	Number of point mutations	Number of DNA copy number variants	Number of deleterious mutations	Normalized mutation rate (mutions/Mb)	Tumor cell lines														
							PA028T	PA086T	PA090T	PA107T	PA122T	PA182T	PA195T	PA215T	PA254T	PA333T	PA018T	PA055T	PA167T	PA202T	PA285T
1	*KRAS*	570	16	7	15	1754.4	S		L	A				A			A	A	A	L	
2	*CDKN2A*	471	1	12	10	1415.4		L	P		L						L				
3	*TP53*	1182	12	14	12	676.8	L	L		L	L	L	L	L	L	L	L	L	L	L	L
4	*SMAD4*	1659	4	11	8	321.5	L					L	L	L	L	L					L
5	***PDCD6***	576	2	4	2	231.5	A							A	A				A		
6	***IL1B***	810	2	1	2	164.6													A		
7	***FAM92B***	915	2	3	2	145.7	A							L					A		
8	*OR4L1*	939	2	3	2	142.0				L		L				L					
9	***SFXN4***	1014	3	1	2	131.5						L									U
10	***RNF207***	1905	3	2	3	105.0	L		L												
11	***ASL***	1395	2	1	2	95.6	A														
12	***TYSND1***	1701	2	2	2	78.4					L				L						
13	***ACSM1***	1734	2	3	2	76.9	L							L					A		
14	***NFE2L2***	1818	2	1	2	73.3													A		
15	*PTPRD*	5739	3	14	6	69.7	P	L	L	P	L	L	L		P	L	L	L	P	L	SL
16	***CCDC41***	2106	2	6	2	63.3			L	L			L	L			L	L			
17	***EXOC8***	2178	2	-	2	61.2															
18	***CLCN4***	2283	2	3	2	58.4	L							L						L	
19	*KIAA1751*	2289	2	1	2	58.2			L												
20	***SOX5***	2292	2	5	2	58.2				A			A	A				A	A		
21	***WDR75***	2493	2	-	2	53.5															
22	***SAGE1***	2715	2	4	2	49.1	L			L								L		L	
23	*GRM8*	2727	2	2	2	48.9					L			L							
24	***TMTC3***	2745	2	8	2	48.6			L	L	L		L	L		L	L	L			
25	***CNTNAP1***	4155	4	3	3	48.1				L			L						L		
26	***ABCC5***	4314	3	1	3	46.4				M			A								
27	***CARD10***	3099	3	5	2	43.0	L			L									L	L	ML
28	***PEAR1***	3114	2	-	2	42.8															
29	***FUK***	3255	2	2	2	41.0												L	A		
30	***SORCS1***	3597	2	6	2	37.1					L	L	L		L		L	L			
31	***GIGYF2***	3963	2	1	2	33.6			L												
32	*CNTNAP2*	3996	2	1	2	33.4															
33	***CLIP1***	4284	2	5	2	31.1			L	L				L			L	L			
34	***MYOM3***	4314	2	3	2	30.9	L		L	L											
35	***LPHN1***	4425	2	4	2	30.1	L			L				L				L			
36	***CDC42BPB***	5136	3	3	2	26.0						L	A			L					S
37	***AKAP11***	5706	2	6	2	23.4	L					A		L			L	L	L		
38	***MYH4***	5820	2	10	2	22.9	L	L				L	L	L		L	L	L	L	L	
39	***EXPH5***	5970	2	1	2	22.3						L									
40	***PHF3***	6120	2	3	2	21.8	L										L	L			
41	***CDC42BPG***	6597	2	4	2	20.2		L		L							A		L		
42	*ARID1A*	6858	2	4	2	19.4	L		L	L						L					
43	***SON***	7281	2	2	2	18.3								L				L			
44	*MLL3*	14736	4	5	4	18.1	A				L			L				L	L		
45	***IGF2R***	7476	2	6	2	17.8	L						L		L		L	L		L	
46	***IGSF10***	7872	2	2	2	16.9							A				A				
47	***UTP20***	8358	2	6	2	16.0			L	L	L		L	L				L			
48	*VWF*	8442	4	4	2	15.8				A			A				A	A			S
49	***AKAP13***	8454	2	1	2	15.8												L			
50	*BRCA2*	10257	3	4	2	13.0	L							L			L	L	L		
51	*ALMS1*	12504	2	1	2	10.7							A								
52	***FAT2***	13050	2	2	2	10.2							L								L
53	***USH2A***	15609	2	1	2	8.5											L				
54	***MACF1***	16293	2	-	2	8.2															
55	***HMCN1***	16908	2	-	2	7.9															
56	*DNAH9*	17896	2	9	2	7.5	L	L				L	L	L		L	L	L			L

#Gene symbol, the genes colored in orange indicate those genes in which mutations have been described previously in PDAC.

Missense Nonsense Synonymous Frame-shift indel

Homozygous deletion (the entire gene) P: Homozygous deletion (part of the gene)

L: LOH A: Amplification M: +1 Missense S: +1 Synonymous U: +1 substitution in UTR

The widely varied mutation rates

Exome-seq revealed that the mutation rates varied significantly among cell lines (Figs. 2C, 3A). The number of somatic substitutions identified from each cell line ranged from 31–640, and the number of somatic indels varied from zero to 100. Accordingly, we classified the cell lines into three subgroups. Cell lines in group 1 ($n = 10$) showed a modest level of somatic mutations, while cell lines in group 2 ($n = 4$) showed a significantly elevated rate of small indels ($P = 0.005$) (Fig. 2C); a cell line in group 3 ($n = 1$) showed dramatically increased rates of both indels and substitutions (Figs. 2C, 3A). In the group-3 cell line, we observed a much higher prevalence of mutations involved in all nine core signaling pathways ($P = 0.0007$) (Supplemental Fig. S6B). In group-2 cell lines, the normalized mutation rate was slightly but significantly increased ($P = 0.037$) in seven of the nine pathways.

Allelic loss of *MLH1* and the increased mutation rate

To find out the genetic factors that accounted for the increased mutation rate in the group-2 and group-3 cell lines, we first screened the MMR genes for somatic alterations. We found that the

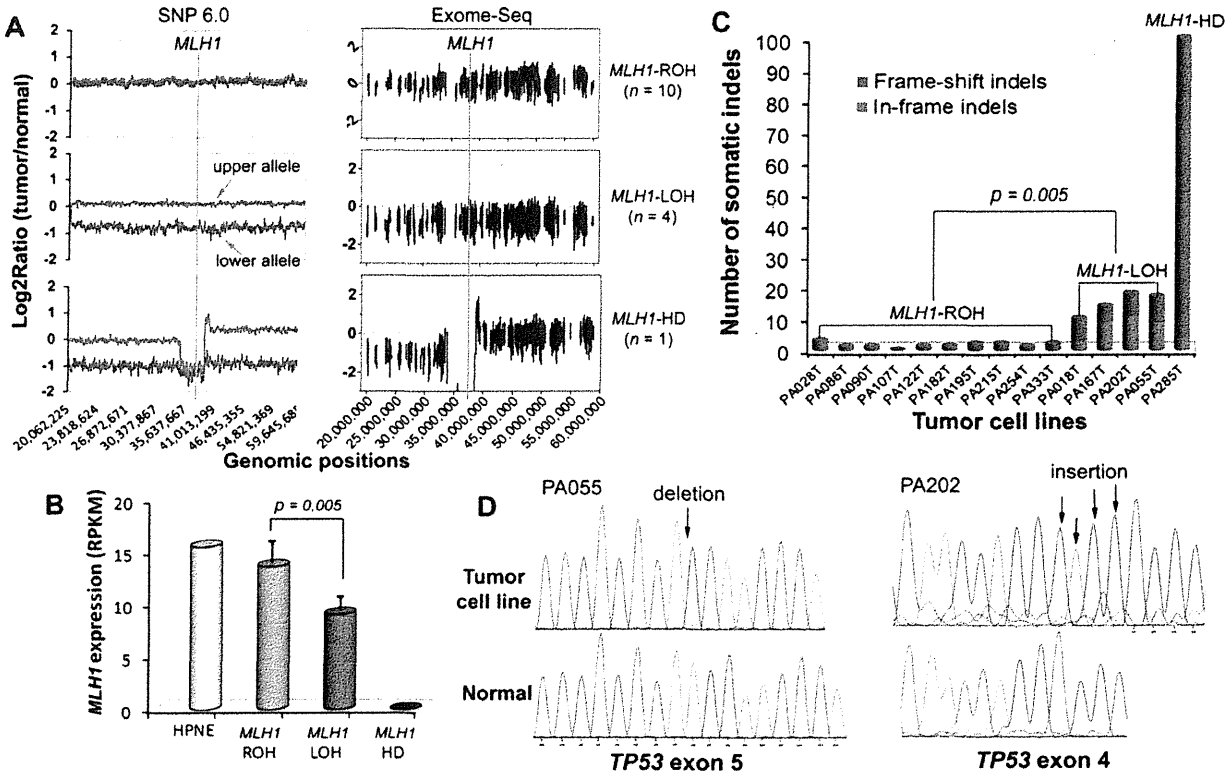

Figure 2. Allelic loss of *MLH1* and the increased rate of somatic indel mutations. (*A*) The distinct DNA copy-number status of *MLH1*. The *left* and *right* panels show the DNA copy-number status inferred from SNP array and Exome-seq data, respectively. The line in light blue indicates the approximate genomic location of *MLH1*. For graphs in the *left* panel, the *y*-axis indicates the adjusted $\log_2$ ratios of signal intensities between the tumor cell line and its matched normal sample for perfect match probes. The red line represents the allele with a higher copy number, and the blue line represents the allele with a lower copy number. The $\log_2$ ratio of −1 and 0 theoretically corresponds to 0 and 1 copy, respectively. For graphs in the *right* panel, the *y*-axis indicates the $\log_2$ ratios of the sequence coverage between the tumor cell line and its matched normal sample for targeted exonic regions. (*B*) The differential expression of *MLH1*. The gene expression level was examined by mRNA-seq. (*RPKM*) Reads per kilobase per million mapped reads. (Bars) Mean ± SD. (*C*) The somatic indels. The number of somatic small indels identified in the targeted exonic regions is shown for each tumor cell line. (*D*) Validation of the truncating indels identified in *TP53* in two *MLH1*-LOH cell lines. (*Left*) 1-bp deletion; (*right*) 4-bp insertion. The positions of indels are indicated by arrows in the sequence electropherograms.

gene *MLH1* was differentially expressed among the subgroups, and the expression levels appeared to be reversely correlated with the mutation rates. As shown in Figure 2B, the expression of *MLH1* decreased by nearly half in group-2 cell lines ($P = 0.005$) and was almost lost in the group-3 cell line. We did not observe any significant differences in the expression of other DNA MMR genes among the subgroups (Supplemental Fig. S9), nor did we detect somatic point mutations of other MMR genes in any of the cell lines. We then quantitatively measured the methylation status of the *MLH1* promoter using MassARRAY, but none of the cell lines showed promoter hypermethylation of this gene (Supplemental Fig. S10). We further examined DNA copy-number changes of *MLH1* and found a clue to its differential expression. As shown in the left panel of Figure 2A, cell lines in group 1 retained both alleles of *MLH1* (*MLH1*-ROH [retention of heterozygosity]), while cell lines in group 2 lost one of the two alleles of this gene (*MLH1*-LOH [loss of heterozygosity]); the cell line in group 3 lost both alleles (*MLH1*-HD [homozygous deletion]). The distinct DNA copy-number status of *MLH1* was also well demonstrated by the read-depth–based Exome-seq data (Fig. 2A, right panel).

Characterization of somatic indels in the *MLH1*-LOH and *MLH1*-HD cell lines

We identified an average of 1.4 ± 0.8 indels per *MLH1*-ROH cell line, 14.8 ± 3.5 indels per *MLH1*-LOH cell line, and 100 indels in the *MLH1*-HD cell line. The mutation rate of the somatic indels was 10.5- and 72.1-fold higher in *MLH1*-LOH and *MLH1*-HD cell lines, respectively, compared with that of the *MLH1*-ROH cell lines ($P = 0.005$) (Fig. 2C). Among the total of 173 somatic indels, 94 were detected in the coding microsatellites (Supplemental Table S1). Prevalence of the indels in the microsatellites was increased sixfold and 154-fold, respectively, in the *MLH1*-LOH and *MLH1*-HD cell lines. Nearly half of the indels that were detected in *MLH1*-LOH cell lines and the majority of indels that were detected in the *MLH1*-HD cell line were frame-shift mutations. Some of the frame-shift indels were present in cancer-related genes such as *TP53*, *BRCA2*, *TGFBR2*, and *MLL3* and were predicted to be protein truncating. We identified a 1-bp insertion in the poly(A)10 tract of *TGFBR2* in one of the *MLH1*-LOH cell lines and validated it by mRNA-seq. We detected two truncating indels in *TP53* in two other *MLH1*-LOH cell lines and validated them by both Sanger sequencing

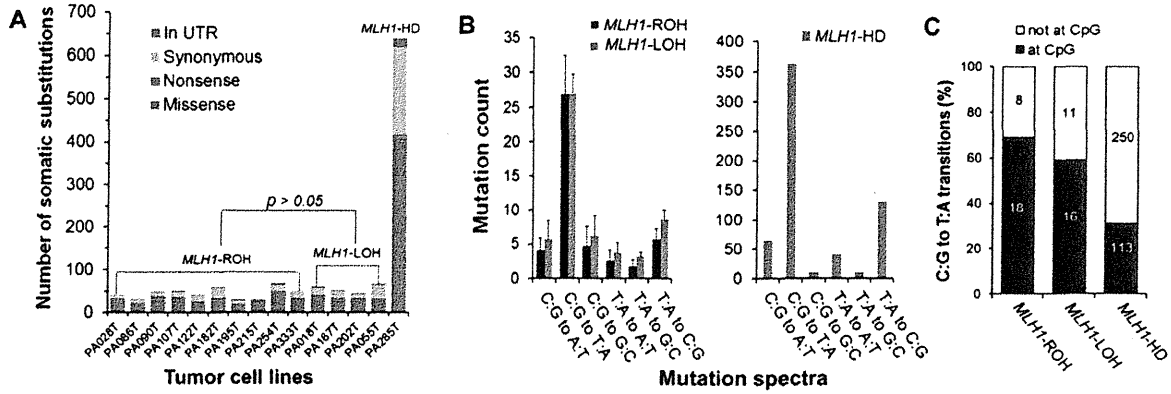

Figure 3. Characterization of the somatic base substitutions. (*A*) The number of somatic base substitutions. The *MLH1*-HD cell line showed a dramatically elevated mutation rate of somatic substitutions. (*B*) The pattern of mutation spectra. (*C*) The distribution of the C:G to T:A transitions at and not at the CpG dinucleotides. For *B*, the data are shown as mean ± SD. As for *C*, the mean values are marked on corresponding columns.

and mRNA-seq (Fig. 2D; Supplemental Table S1). Both indels were accompanied by LOH and introduced premature termination codons (PTCs), resulting in a dramatic reduction of *TP53* expression (Supplemental Fig. S11).

The mutation spectra

The pattern of mutation spectra was quite similar among the subgroups. As shown in Figure 3B, the predominant type of base substitution was the C:G to T:A transition, followed by the T:A to C:G transition. Many cancer genes such as *KRAS*, *TP53*, *SMAD4*, and *APC* were mutated by a C:G to T:A transition. In the *MLH1*-HD cell line, the mutation rate of the C:G to T:A transitions was markedly increased, especially at non-CpG sites (Fig. 3C). The frequency of other classes of base substitution was also dramatically higher except for the C:G to G:C and T:A to G:C transversions.

Evaluation of genomic instability using Exome-seq

Based on the Exome-seq data, we determined the microsatellite instability (MSI) status of *MLH1*-ROH, *MLH1*-LOH, and *MLH1*-HD cell lines as "stable," "intermediately unstable," and "highly unstable," respectively (Supplemental Table S1). We then performed the conventional MSI assay for the same sample set (Supplemental Fig. S12). The assay revealed that all seven markers were stable in the *MLH1*-ROH cell lines, and two of the markers, D17S250 and D2S123, were unstable in the *MLH1*-HD cell line. However, none of the markers showed instability in any of the *MLH1*-LOH cell lines. Using the conventional MSI assay, *MLH1*-LOH cell lines were indistinguishable from *MLH1*-ROH cell lines. To further evaluate the performance of Exome-seq, we selected three representative coding microsatellites, within which somatic indels have been identified by Exome-seq and validated by mRNA-seq. We designed fluorescence-labeled primers and performed the MSI assay. The conventional assay confirmed instability for all three microsatellites (Fig. 4).

Discussion

In this study, we analyzed 15 PDAC-derived cell lines and their matching normal tissues using Exome-seq. We detected more than 1500 point mutations and showed that 1359 genes were somatically altered in at least one of the cell lines. *KRAS*, *TP53*, *CDKN2A*, and *SMAD4*, known as the "master" genes for PDAC, were the top four most frequently mutated genes identified in this study. These results are consistent with an early study performed by Jones and colleagues (2008) using the Sanger sequencing method, indicating a good performance of Exome-seq, as well as our mutation detection pipeline.

Mutation of the four key players, although being of paramount importance, may not be sufficient to drive the development and progression of PDAC, since variability can occur among tumors arising in the same organ and among cell populations within the same tumor. Recent studies have reported the intertumoral heterogeneity among PDACs and the intratumoral heterogeneity in a hepatocellular carcinoma (Kim et al. 2011; Totoki et al. 2011). The number of mutated genes that drive development of cancer was found to be far greater than previously thought (Greenman et al. 2007). By using Exome-seq, we identified additional 52 genes that recurrently mutated in PDAC. Among them, the mutation of 41 genes has not been described in this cancer type. More than half of these genes have been suggested to play a role in carcinogenesis. For example, a recent study showed *NFE2L2* is frequently mutated in lung cancers (Shibata et al. 2010). The overexpression of *SOX5* is associated with prostate tumor progression and early development of distant metastasis (Ma et al. 2009). *EXOC8* has been shown to foster oncogenic Ras-mediated tumorigenesis (Issaq et al. 2010). Mutation screening of these genes in a large sample size would help us gain a further understanding of their biological contribution to PDAC.

The application of NGS technologies to cancer genomics has dramatically increased the efficiency of mutation discovery. Since a variety of factors, such as sequencing platforms, data mapping, and variant calling algorithms can affect the final output of identified mutation candidates, validation of the numerous proposed mutations has consequently become a common issue to be considered. We here evaluated the performance of mRNA-seq in verification of mutations identified in coding regions. If we simply consider all somatic mutations identified by Exome-seq, 61.6% of them were validated by mRNA-seq. If we focus, however, on those mutations in expressed genes, 94.3% of them can be successfully confirmed by mRNA-seq. For truncating mutations, despite a lower abundance of the mutant allele in cDNA, mRNA-seq was still able to confirm 86.2% of the mutations. This suggests that although it may miss mutations in poorly expressed regions, mRNA-seq may

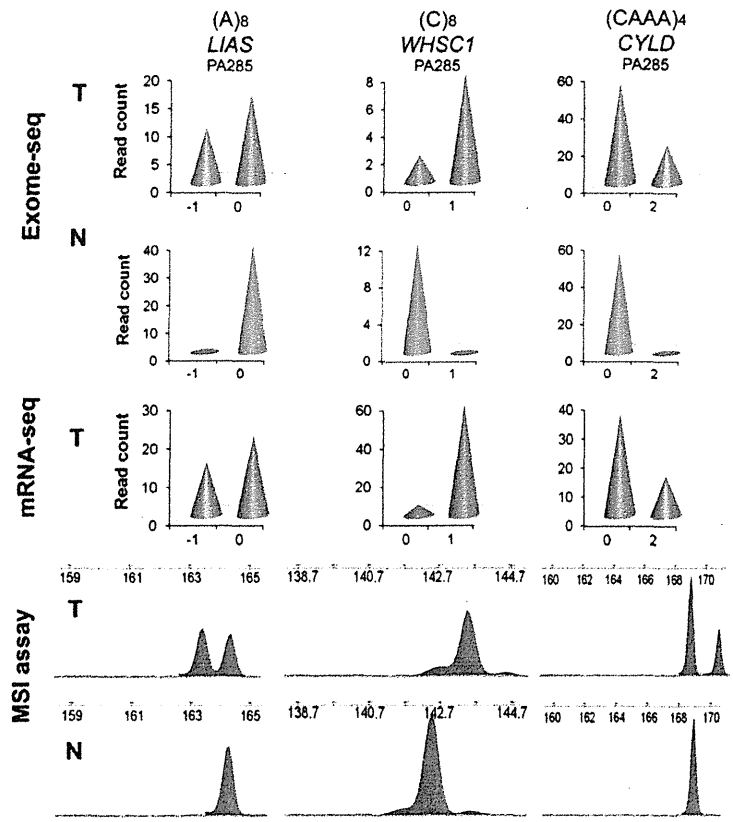

Figure 4. MSI analysis using Exome-seq. The data for three representative microsatellites are shown. (*Top*) Read-depth based Exome-seq data; (*middle*) mRNA-seq data; (*bottom*) electropherograms of the conventional MSI assay. For the *top* and *middle* panels, the *x*-axis indicates the lengths of indels. The negative value indicates base deletion, and the positive value indicates base insertion, while 0 indicates no indel. The numbers marked at the *y*-axis indicate the number of sequence reads that carry the mutant allele or the wild-type allele. (*Bottom*) *x*-axis is the size in bases; *y*-axis is the fluorescence intensity. The red peaks are internal size standards.

be a workable alternative to Sanger sequencing for the validation of mutations identified in expressed genes. In addition to learn about gene expression and splicing variants, groups who run NGS on both gDNA and cDNA for the same sample set may get an extra benefit from such an application.

Allelic loss at the short arm of chromosome 3 is one of the most common genetic alterations observed in human cancers. It has been reported in over 30% of PDAC and nearly 90% of RCC cases (Yamano et al. 2000; Harada et al. 2008; Toma et al. 2008). Many potential cancer genes have been identified on chromosome 3p. The DNA MMR gene *MLH1* is located at chromosome 3p22.2. In mammals, the MLH1 protein is an essential component of the MMR complex. MLH1 protein binds to either PMS1 or PMS2, and both heterodimers bind either to the MSH2/MSH6 heterodimers to correct mismatches or to the MSH2/MSH3 heterodimers to correct indel errors (Jiricny 1998; Kolodner and Marsischky 1999; Raschle et al. 1999). Among the MMR proteins, the loss of MLH1 is by far the most common cause of MSI. To date, a variety of genetic and epigenetic alterations in *MLH1* has been discovered in many different types of cancers (Bronner et al. 1994; Cunningham et al. 1998; Kuismanen et al. 2000; Suter et al. 2004; Arnold et al. 2009). In pancreatic cancers, the mutation of *MLH1* and MSI has been reported in a histologically distinct subset of poorly differentiated adenocarcinomas, called medullary carcinomas, which usually have a wild-type *KRAS*. The sporadic PDAC, however, seldom, if ever, has MSI (Wilentz et al. 2000). To our knowledge, the profile of MSI has yet to be fully demonstrated in a genome-wide manner in pancreatic cancers.

Homozygous deletion of *MLH1* is a rare case and has not been documented previously. In one of the cell lines analyzed in this study, we incidentally detected a focal homozygous deletion spanning the entire *MLH1* locus. Exome-seq revealed intensive genomic instability in this cell line, indicated by a dramatically elevated mutation rate of somatic substitutions, small indels, as well as the indels presented in coding microsatellites. The number of C:G to T:A transitions was markedly increased, especially at non-CpG sites, suggesting an impaired recognition/repair of G:T mismatches (Marra and Schar 1999; Kumar et al. 2009). The mutation spectrum of the cell line was quite similar to that of other types of MMR-deficient tumors previously reported (Greenman et al. 2007).

Although allelic loss of *MLH1* has been reported in over 30% of PDACs (Yamano et al. 2000; Harada et al. 2008), no statistical correlation has been described between *MLH1* allelic loss and an increased mutation rate. It was previously thought that mutations in *MLH1* and other DNA MMR genes are recessive; i.e., a single copy of the wild-type *MLH1* allele is sufficient to perform its normal function (Bodmer et al. 2008; Negrini et al. 2010). In this study, we notably found that *MLH1* expression was decreased by nearly half in cell lines with an allelic loss of *MLH1*. While these cell lines were negative in a conventional MSI assay, they showed a 10.5-fold increase in the rate of somatic indels. We also observed a higher prevalence of indels in the coding microsatellites. Moreover, we identified truncating indels that inactivate tumor suppressor genes, such as *TP53* and *TGFBR2*. These results indicate that deletion of one copy of *MLH1* gene results in haploinsufficiency in the correction of DNA indel errors.

An earlier study performed in vitro could support our argument that hemizygous deletion of *MLH1* may lead to an impaired DNA repair and genomic instability. Edelmann and colleagues (1996) generated mice with a null mutation of the *MLH1* gene and measured the MMR activity in vitro using the cell-free extracts from the mouse embryo-derived fibroblast (MEF). They found that the embedded errors in the reporter gene were repaired 2.3-fold less efficiently in MEF extracts of $mlh1^{+/-}$ mice compared with that of $mlh1^{+/+}$ mice.

To further address the significance of *MLH1* hemizygous deletion in in vivo tumors, we examined the primary RCC samples, which usually exhibit LOH on chromosome 3p. All patients provided informed consent for the research use of their samples, and

the study was approved by the institutional review board of the National Cancer Center Research Institute. We enriched the exonic sequences of 15 primary RCCs and their matched normal samples using the Agilent Human All Exon 50 Mb Kit and sequenced the exomes using the HiSeq 2000 sequencing system. Among the 15 RCCs analyzed, 13 cases showed LOH at the *MLH1* locus on chromosome 3p, and two cases showed ROH. The data are shown in Supplemental Figure S13 and Supplemental Table S3. On average, we identified 1.5 somatic indels in the *MLH1*-ROH cases, which is consistent with a previous report (Varela et al. 2011). However, in the *MLH1*-LOH tumors, we observed a 4.6-fold increased rate of somatic indel mutations ($P = 0.0008$). A total of 90 somatic indels were identified in 13 *MLH1*-LOH cases. Among them, 85 were frame-shift indels and 68 were truncating indels. Moreover, we detected recurrent truncating indels in several well-characterized cancer genes, such as *VHL* (four cases), *PBRM1* (four cases), and *JARID1C* (four cases). These data suggest that the correlation we observed between *MLH1* allelic loss and the increased mutation rate of somatic indels is more likely to be the true rather than a simple coincidence. Our data also indicate that *MLH1* allelic deletion, through increasing the frequency of somatic indel mutations in cancer genes, could drive the development and progression of cancer. It is potentially significant that the correlation we observed was only with somatic indels, and not base substitutions. Presumably, MLH1 protein may play a pivotal role in correction of DNA indel errors, while its function for MMR can be partially compensated by other MMR proteins or mechanisms. Nevertheless, we could not exclude the possibility that factors that predispose to DNA copy-number losses might also associate with indel frequency.

In human cancers, LOH at chromosome 3p is frequently observed (Yamano et al. 2000; Harada et al. 2008; Toma et al. 2008). However, the association between *MLH1* allelic loss and the increased rate of somatic indel mutations has not been notified. There are several possible reasons. First, depending on the platform, sequencing indels can be difficult. Second, reads arising from indel sequence are generally more difficult to be aligned to the reference genome. Without a good coverage, indels are more difficult to be detected. Third, the MSI assay is conventionally used to evaluate the occurrence of indels at microsatellites as genome-wide mutation analysis was not available until recently (Boland et al. 1998). The MSI assay is insufficient since only several microsatellites are selected. In addition, technical limits exist in the conventional assay (Hatch et al. 2005; Fujii et al. 2009). For example, the assay system employs capillary electrophoresis and autoradiography, making it sometimes difficult to recognize small changes in the microsatellite sequences. Some artificial fragment peaks were usually introduced after 32 cycles of PCR amplification. The choice of markers may also affect the sensitivity of the assay (Hatch et al. 2005; Fujii et al. 2009). In contrast, our data suggest that Exome-seq may be an acceptable alternative for microsatellite analysis.

Methods

The samples

PDAC-derived cell lines

We analyzed a total of 15 PDAC-derived cell lines and their matched normal samples. Primary pancreatic tumor tissue contains a high admixture of contaminating non-neoplastic inflammatory and stromal cells. To remove the non-neoplastic cells and facilitate the detection of somatic mutations, microdissected primary tumors were passaged in vitro as cell lines prior to extracting DNA and RNA for sequence analysis. The characteristics of the PDAC-derived cell lines are listed in Table 1. All cell lines were established by researchers at the Cancer Institutes, Japanese Foundation of Cancer Research (JFCR). The matching normal tissues were surgically resected from tumor-negative pancreas. All normal samples were histologically reviewed by two pathologists and were confirmed to be free of tumor tissues. All patients provided informed consent for the research use of their samples, and the study was approved by the institutional review board of the JFCR and the University of Tokyo. The DNA and RNA were extracted by standard protocols. The pair matching of each tumor cell line and the normal sample was confirmed by genome-wide SNP array (Affymetrix).

HPNE cell line

The human telomerase reverse transcriptase (hTERT)-immortalized pancreas duct epithelial cell line (hTERT-HPNE, CRL-4023) was purchased from The American Type Culture Collection (ATCC). The cells were cultured in low-glucose DMEM media (Invitrogen) supplemented with 25% Medium M3 Base (Incell), 5% fetal bovine serum, and 10 ng/mL human recombinant epithelial growth factor (Sigma Aldrich) at 37°C and with 5% carbon dioxide. HPNE serves as the normal control for gene expression analysis.

Exome-seq and data analysis

Exome-seq

Targeted enrichment was performed with Agilent SureSelect Human All Exon Kit V1.0 (Agilent Technologies). This kit is designed to enrich 162,073 exons of 16,954 protein-coding genes, more than 700 microRNAs and 300 noncoding RNAs, covering ~37.6 Mb of the human genome (Supplemental Fig. S14). SureSelect Biotinlyated RNA baits were designed to be 120-mer long and end-to-end tiled (1× tilling). The gDNA libraries were prepared using an Illumina paired-end DNA sample prep kit (Illumina) following the manufacturer's protocols with slight modifications. In brief, 3 μg gDNA was fragmented using Covaris Acoustic Solubilizer (Covaris) with 20% duty cycle, 4 intensity, and 200 cycles per burst for 160 sec, at 16°C to get DNA fragments with a mean size of 200 bp. Fragmented DNA was then purified using Agencourt AMPure XP magnetic beads (Beckman Coulter). The concentration of the library was measured using a Bioanalyzer (Agilent Technologies). The adapter-ligated libraries were amplified with six PCR cycles, and 500 ng of each amplified library was hybridized with Biotinlyated RNA baits in solution for 24 h for target enrichment. Subsequently, hybridized libraries were cleaned up and further amplified with 12 cycles of PCR; 5–6 pM/lane DNA was applied to the flow cell, and paired-end 76-nucleotide (nt)-long reads were generated using the Illumina Genome Analyzer IIx Platform (GAIIx). Each sample was run on a single lane of Illumina flow cell except for samples PA028N and PA167T, which were each run on two lanes.

Data alignment and variant calling

The detail workflow for data alignment and mutation detection was described in Supplemental Figure S15. For each cell line and matched normal sample, the sequence reads were mapped to the human NCBI Build 36 reference sequence (hg18, downloaded from http://genome.ucsc.edu) initially with the Illumina sequencing pipeline (version 1.6) for quality recalibration. The passing filter (PF) reads were then mapped again using BWA (version 0.5.8) (Li and Durbin 2009). Any potential PCR duplicates, ambiguous reads, inconsistent read pairs, and singletons were excluded. Only the unique reads that mapped in consistent read pairs (with proper insert size and orientations) were selected for further

analysis. The bases substitutions were called using SAMtools (version 0.1.7) (Li et al. 2009), and the indels were called using both SAMtools and Pindel algorithms (Ye et al. 2009).

Variant filtering and somatic variant identification

To pick out the high-confident somatic variants, we applied the following rigorous filters and rules to the data set (Supplemental Fig. S15). The first filter applied is the "quality filter." Variants with a mapping quality of 20 or more, a *phred*-like consensus quality of 20 or more, a base call quality of more than 10, and a sequence coverage of 10× or more for both the cell line and matched normal sample were considered as high-quality variants. The setting for the filter conditions were optimized by comparing common SNPs detected by BWA (Li and Durbin. 2009) with those genotyped using Affymetrix Human SNP Array 6.0 (Affymetrix), ensuring a high concordance (99.84%) across two analyses (Supplemental Fig. S16).

The second filter applied, referred to as the "somatic filter," seeks to pick out the somatically acquired variants. All the high-quality variants produced from the above steps were passed through the "somatic filter," and only those meeting the threshold were considered as the somatic variants. The mutant allele (nonreference allele) ratio was calculated as follows:

$$\textit{Mutant allele ratio} = \frac{\text{Count of non-reference bases}}{\text{Count of total bases}} \times 100\%$$

The setting for the "somatic filter" is as described in Supplemental Figure S15; for the cell line sample, it is required that four or more reads supporting the mutant allele and the mutant allele ratio should be 15% or more. Moreover, the mutant allele should be supported by reads that aligned in both the forward and reverse directions. For the matched normal sample, given the potential sequencing errors and mapping errors, the mismatch should not be detected in more than 3% of the aligned reads and should not be detected in more than two reads. The indel, however, should not be detected in any of the aligned reads.

The third filter, referred to as the "false-positive filter," was then applied. This filter is used to remove the potential false-positive events that result from the homologous sequences within the human genome, mapping errors, and so on. For each of the somatic mutations produced in the above steps, we extracted 200–300 bases of DNA sequences flanking its mutation locus and mapped the sequences to hg18 using the BLAT algorithm. Subsequently, the mutations identified within the regions rich for homologous sequences were removed from the list. The somatic mutations were further examined using the integrated genome viewer (IGV), and any mutations found in a "noisy" background (multiple mismatches or indels in flanking sequences) were removed from the list.

As for detection of indels, one more step, called "rescue," was applied since the sequence read carrying a long indel toward its end is usually difficult to be aligned properly. We use the Pindel algorithm to rescue those possibly missed indels.

Variant annotation

Functional effects of filtered somatic variants were predicted using the SIFT algorithm (Kumar et al. 2009; http://sift.jcvi.org). The SIFT algorithm predicts whether an amino acid substitution affects protein function based on sequence homology and the physical properties of amino acids.

Mutation rate calculation and normalization

The background mutation rate (mutations/per Mb coding sequences) was calculated as follows:

$$\frac{\text{Sum of somatic mutations}}{\text{Sum length of exome targets} \times \text{number of tumor cell lines}}$$

The mutation rate of each gene was normalized by the frequency of mutations and the length of its coding sequences. Only somatic deleterious mutations, including missense substitutions, nonsense substitutions, frame-shift indels, and focal homozygous deletions were counted. The normalized mutation rate for each gene was calculated as follows, and a priority list was made accordingly:

$$\frac{\text{Sum of somatic mutations identified in the gene}}{\text{Sum length of coding regions of the gene} \times \text{number of tumor cell lines}}$$

Pathway analysis

The genes with somatic mutations were classified into different functional pathways using the Gene Ontology (GO) database (http://www.geneontology.org/). Only somatic deleterious mutations were counted. The normalized mutation rate for each pathway was calculated as below:

$$\frac{\text{Sum of somatic mutations identified in genes included}}{\text{Sum length of coding regions of genes included} \times \text{number of tumor cell lines}}$$

mRNA-seq and data analysis

Library preparation and mRNA-seq

Total RNA was extracted from PDAC-derived cell lines and the HPNE cells using the protocol outlined in the RNeasy Kit (Qiagen). Total RNA integrity was measured using a 2100 Bioanalyzer (Agilent Technologies), and all samples were confirmed to have an RNA Integrity Number (RIN) greater than 8.0 prior to further analysis. The mRNA-seq libraries were prepared using a paired-end mRNA Sequencing Sample Prep Kit (Illumina) following the manufacturer's protocols with slight modifications. Briefly, 2 μg of total RNA was used as the starting material, and the polyadenylated RNAs were selected using Sera-Mag Magnetic Oligo(dT) Beads (Illumina). The Poly(A)$^+$ RNA was then fragmented by heating for 90 sec at 94°C in the supplied fragmentation buffer. Fragmented RNA was mixed with random primers, incubated for 5 min at 65°C, and placed on ice briefly before starting cDNA synthesis. First-strand cDNA synthesis was performed using SuperScript II, and second-strand cDNA synthesis was performed using DNA Pol I in the supplied GEX second-strand reaction buffer. Subsequently, cDNA ends were repaired, and adenine was added to the 3′ end of the cDNA fragments to allow adaptor ligation. Paired-end adaptors were ligated to the cDNA fragments. The ligated product was run on a 2% agarose gel, and a 300 ± 20 bp fragment was cut out and extracted. PCR (eight cycles) was performed with Phusion High-Fidelity DNA Polymerase (Finnzymes Oy) following the manufacturer's protocols. The PCR products were cleaned up with Agencourt AMPure XP magnetic beads (Beckman Coulter); 6.0–6.7 pM/lane cDNA was applied to the flow cell and paired-end 76-nt-long reads were generated using Illumina GAIIx. Each sample was run on two lanes of Illumina flow cell.

Data alignment

All PF reads were aligned to hg18 using TopHat spliced aligner (Trapnell et al. 2009). Meanwhile, all PF reads were aligned to NCBI Reference Sequence (RefSeq) mRNA sequences using BWA. A merged file was generated for each sample by integrating the output of

TopHat with that of BWA for an optimal alignment for each sequence read. The ambiguously mapped reads and the duplicates were excluded. The level of gene expression was calculated in reads per kilobase of exonic sequence per million aligned reads (RPKM).

Mutation validation

For each of the somatic mutations identified by Exome-seq, we extracted the aligned mRNA-seq reads at its corresponding locus and examined if the mutant allele was also present in the cDNA sequences. The substitutions were called using SAMtools (Li et al. 2009). The small indels were called by both the SAMtools and Pindel algorithms (Ye et al. 2009). We focus on those loci covered by at least five reads, since it is rather difficult to call the variant accurately for poorly expressed genes. The mutation is supposed to be verified by mRNA-seq if at least two reads carried the mutant allele, and the mutant allele was detected in no less than 5% of the total reads aligned. For those loci covered by less than five reads but two or more reads, the mutation was also supposed to be verified if at least two reads carried the mutant allele.

Genome-wide SNP genotyping and DNA copy-number analysis

Genome-wide SNP genotyping was performed using the Affymetrix Genome-wide Human SNP Array 6.0 (Affymetrix) according to the manufacturer's instructions. SNPs were genotyped using the Birdseed version 2 module of the Affymetrix Genotyping Console software GTC 4.0.1, together with data from 45 HapMap-JPT samples (CEL files obtained from Affymetrix). DNA copy-number changes were analyzed using the Genome Imbalance Map (GIM) algorithm, as we previously described (Ishikawa et al. 2005).

The conventional MSI assay

The conventional MSI assay was performed using the proposed "Bethesda" panel of fluorescence-labeled markers, including *BAT25*, *BAT26*, *D2S123*, *D5S346*, and *D17S250* and an additional two markers, *NR21* and *NR27*. The primer sequences and PCR conditions have been previously described (Murayama-Hosokawa et al. 2010). In this study, we selected an additional three coding microsatellites and designed 6-carboxyfluorescein–labeled primers. Sequences of oligonucleotide primers for these three microsatellites are listed in Supplemental Table S4. PCR reactions were performed using the previously described reagents (Murayama-Hosokawa et al. 2010) under the following thermal cycle conditions: initial denature for 2 min at 94°C, followed by 32 cycles of denature for 15 sec at 94°C, annealing for 30 sec at 58°C, and primer extension for 30 sec at 68°C; the final extension step was carried out for 2 min at 68°C. After PCR, 1 μL of the properly diluted PCR product was mixed with 10 μL of Hi-Di Formamide and GeneScan 500 LIZ Size Standard (Applied Biosystems) mixture (37:1). This product was then denatured for 5 min at 95°C and put on ice immediately for 5 min before loading onto ABI 3130xl Genetic Analyzer (Applied Biosystems). The output data files were analyzed by GeneMapper Software Version 4.0 (Applied Biosystems). Determination of MSI status was made according to the presence of mutant alleles in tumor DNA compared with matched normal DNA.

MSI analysis by Exome-seq

We established a data analysis pipeline to identify small indels in the microsatellites. For each of the somatic indels identified in this study, we extracted the 50 bases of DNA sequences flanking its locus and examined if the indel was present in microsatellite sequences. Only those indels detected in the protein-coding microsatellites with at most 6 nt and repeated at least five times for mono- and dinucleotide microsatellites and at least three times for multiple-nucleotide microsatellites were counted. As shown in Figure 4, a graph was plotted for the indels in coding microsatellites according to the lengths of the indels and the number of sequence reads that supported the mutant alleles or the wild-type alleles. The microsatellite was suggested to be instable if a shorter allele (deletion) or a longer allele (insertion) was detected only in the tumor DNA. The sequence homology of each supporting read was further examined by the BLAT algorithm, and the reads rich of homologous sequences were discarded. The mutant allele ratio was then calculated using a formula as mentioned above.

MLH1 promoter methylation analysis

The methylation status of *MLH1* promoter was quantitatively measured using MassARRAY (Sequenom), as previously described (Yagi et al. 2010). Briefly, 500 ng gDNA was bisulfite converted using an EZ DNA Methylation Kit (Zymo Research) according to the manufacturer's instruction manual. Bisulfite-treated DNA was PCR amplified, and the PCR product was transcribed by in vitro transcription (IVT) prior to cleavage using RNase A. Unmethylated cytosine was converted to uracil by bisulfite treatment, while the methylated cytosine was not converted. Methylation status was then determined by the mass difference between A and G in the cleaved RNA product. Quantitative methylation scores were obtained at each analytic unit of a cleaved product, referred to as "CpG unit." The amplified DNA that was not methylated at all in any CpG sites was used as an unmethylated (0%) control. The amplified DNA, methylated by SssI methylase, was used as a fully methylated (100%) control.

Sanger sequencing

Oligo primers were designed to amplify the genome fragments containing the candidate nucleotide mutations from tumor cell line DNA and the matched normal DNA. PCR was performed using the high-fidelity DNA polymerase KOD-plus (TOYOBO) under optimized thermal conditions. PCR products were evaluated on a 2% agarose gel, purified and sequenced in both directions using Big Dye Terminator reactions, and subsequently loaded on an ABI 3130xl capillary sequencer (Applied Biosystems).

Statistical analysis

The *P*-value was calculated by Student's *t*-test when the data were normally distributed or by the nonparametric Wilcoxon signed-rank test when the data were not normally distributed. *P*-values less than 0.05 were considered to be statistically significant.

Data access

The Exome-seq data, mRNA-seq data, and SNP array data have been submitted to the European Genome-Phenome Archive (EGA; http://www.ebi.ac.uk/ega/), which is hosted at the European Bioinformatics Institute (EBI), under accession no. EGAS00001000149.

Acknowledgments

We thank Dr. Teruhiko Yoshida for having organized collaboration on the Exome-seq of the RCC project. We thank Ms. Kaori Shiina, Ms. Hiroko Meguro, Ms. Kaoru Nakano, and Ms. Saori Kawanabe for their excellent technical assistance. We acknowledge Dr. Michael Jones for the critical reading of the manuscript. This study was supported by Grants-in-Aid for Scientific Research (H.A.) and Scien-

tific Research on Priority Areas (H.A., T.N.); a grant for Translational Systems Biology and Medicine Initiative (TSBMI; H.A.) from the Ministry of Education, Culture, Sports, Science and Technology; the NFAT project from the New Energy and Industrial Technology Development Organization (NEDO; H.A., T.N.), Japan; and the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO; T.S., H.S.,Y.K.).

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Received March 9, 2011; accepted in revised form October 3, 2011.

Int. J. Radiation Oncology Biol. Phys., Vol. 79, No. 5, pp. 1479–1486, 2011

0360-3016/$–see front matter

ELSEVIER

doi:10.1016/j.ijrobp.2009.12.048

CLINICAL INVESTIGATION **Liver**

DOSE–VOLUME HISTOGRAM ANALYSIS OF THE SAFETY OF PROTON BEAM THERAPY FOR UNRESECTABLE HEPATOCELLULAR CARCINOMA

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Purpose: To evaluate the safety and efficacy of radiotherapy using proton beam (PRT) for unresectable hepatocellular carcinoma.

Methods and Materials: Sixty consecutive patients who underwent PRT between May 1999 and July 2007 were analyzed. There were 42 males and 18 females, with a median age of 70 years (48–92 years). All but 1 patient had a single lesion with a median diameter of 45 mm (20–100 mm). Total PRT dose/fractionation was 76–cobalt Gray equivalent (CGE)/20 fractions in 46 patients, 65 CGE/26 fractions in 11 patients, and 60 CGE/10 fractions in 3 patients. The risk of developing proton-induced hepatic insufficiency (PHI) was estimated using dose-volume histograms and an indocyanine-green retention rate at 15 minutes (ICG R15).

Results: None of the 20 patients with ICG R15 of less than 20% developed PHI, whereas 6 of 8 patients with ICG R15 values of 50% or higher developed PHI. Among 32 patients whose ICG R15 ranged from 20% to 49.9%, PHI was observed only in patients who had received 30 CGE (V30) to more than 25% of the noncancerous parts of the liver ($n = 5$) Local progression-free and overall survival rates at 3 years were 90% (95% confidence interval [CI], 80–99%) and 56% (95% CI, 43–69%), respectively. A gastrointestinal toxicity of Grade ≥2 was observed in 3 patients.

Conclusions: ICG R15 and V30 are recommended as useful predictors for the risk of developing PHI, which should be incorporated into multidisciplinary treatment plans for patients with this disease.

Hepatocellular carcinoma, Proton beam radiotherapy, Dose–volume histogram, Radiation tolerance of the liver.

INTRODUCTION

Recent improvements in diagnostic imaging and radiotherapy (RT) techniques have made high-dose radiotherapy a safe and effective treatment for selected patients with unresectable hepatocellular carcinoma (HCC) (1). Charged-particle radiotherapy can potentially deliver considerably larger doses of RT to liver tumors, with greater sparing of normal tissues, and proton beam radiotherapy (PRT) for HCC using aggressively high total and fractional RT doses has been investigated during the last 2 decades. The results have shown local control rates ranging from 75% to 96% and overall survival (OAS) rates exceeding 50% at 2 years in groups of patients that include those who had HCC tumors of ≥5 cm in diameter (2–4). HCC has a high propensity for venous invasion, which is frequently associated with multiple tumors within resected specimens (5–9). In this context, the extent of resection was determined while considering potential tumor spread via portal blood flow and the necessity of preserving a functional liver reserve (5, 7, 10). Even in preselected patients who underwent hepatectomy, more than 50% of tumors with diameters greater than 4 cm demonstrated microscopic vascular invasion (8, 11). Consequently, it will become more crucial to consider the influence of vascular invasion on undetectable tumor dissemination at the periphery of the gross tumor in RT for unresectable HCC.

Given the high probability of obtaining local control by using PRT, an appropriate definition of the clinical target volume (CTV) according to patterns of tumor spread and patients' functional liver reserves is extremely important in order to maximize the therapeutic ratio. Ideally, the entire portal segment that contains HCC nodules should be covered within the CTV when the tumor shows macro- or microscopic vascular invasion. This requires a considerably larger

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Conflict of interest: none.

Received Nov 24, 2009, and in revised form Dec 21, 2009. Accepted for publication Dec 21, 2009.

irradiated volume even with PRT, partly because of unavoidable uncertainty in treatment planning without using intraoperative ultrasonography (7). Another possible way to eradicate satellite HCC nodules, which are disseminated via portal blood flow, is transarterial chemoembolization (TACE). Currently, the standard treatment for patients with unresectable HCC that is not amenable to local ablation therapy is TACE instead of best supportive care (12). The OAS rate at 3 years after TACE ranges from 32% to 47% in patients with stage III cancer and with liver damage A to B, according to the staging system used in a nationwide cohort study conducted by the Liver Cancer Study Group of Japan (13). Considering that the tumoricidal effect of TACE in HCC with vascular invasion is frequently incomplete (13), a significant benefit of adding PRT to TACE would be expected. However, presently, there has been no robust evidence supporting this concept. Before we examine the validity of targeting the entire anatomical portal segment containing HCC in a multidisciplinary approach that includes PRT, practical methods to estimate the safety of PRT according to the dose–volume histogram (DVH) should be established in patients who have various levels of severity of liver dysfunction. Findings from our previous study consisting of 30 patients suggested that the risk of proton-induced hepatic insufficiency (PHI) could be predicted by the indocyanine green clearance test and the retention rate at 15 minutes (ICG R15) in combination with DVH parameters (14) such as percentages of hepatic noncancerous portions receiving doses of >30 cobalt-Gray-equivalent (CGE) (3). We have subsequently accumulated data from additional patients in clinical practice. The clinical results were evaluated, and we have again used the DVH analysis to examine the relationship between probability of PHI and dose–volume parameters.

METHODS AND MATERIALS

Patients

Patient eligibility was reported previously (3); in brief, they were required to have uni- or bidimensional measurable HCC nodules of ≤10 cm in maximum diameter on computed tomography (CT) and/or magnetic resonance imaging (MRI) without evidence of extrahepatic tumor spread. All patients had a white blood cell count of ≥2,000/mm^3; a hemoglobin level of ≥7.5 g/dl; a platelet count of ≥25,000/ mm^3; and adequate hepatic function (total bilirubin, ≤3.0 mg/dl; alkaline phosphatase, aspartate aminotransferase, and alanine aminotransferase of <5.0× normal; no ascites). Patients who had multicentric HCC nodules were not considered as candidates for PRT, except for those who fulfilled the following two conditions: (*1*) multiple nodules could be encompassed within a single clinical target volume; and (*2*) lesions other than those of the targeted tumor were judged to be controlled with prior surgery and/or local ablation therapy. This retrospective study was approved by the institutional ethics committee, and written informed consent was obtained from all patients.

Treatment Planning

ICG R15 was measured in all patients to quantitatively assess the hepatic functional reserve. Serological testing for hepatitis B surface antigen and anti-hepatitis C antibody was done. All patients were judged to be unresectable by expert hepatobiliary surgeons at our institution, based on the patient's serum bilirubin level, ICG R15, and expected volume of resected liver (10). Percutaneous fine-needle biopsies were performed for all patients unless they had radiologically compatible, postsurgical recurrent HCC (3).

Treatment methods were published previously (3). In brief, gross tumor volume (GTV) was defined using a treatment-planning CT scan, and CTV and planning target volume (PTV) were defined as follows in all but 2 patients: CTV = GTV + 5 mm, and PTV = CTV + 3 mm of lateral, craniocaudal, and anteroposterior margins. CTV encompassed the entire volume of the right lobe in 1 patient who had a tumor of 4 cm in diameter that broadly attached to the bifurcation of the right anterior and posterior portal veins. In this patient, right portal vein embolization was done to facilitate compensatory hypertrophy of the left lobe for expected surgery. However, the patient was finally judged to be unresectable, and PRT was selected. Another patient was treated with a CTV encompassing the entire right anterior portal segment because a tumor of 2 cm in diameter had invaded the bifurcation of the right anterosuperior and anteroinferior portal vein associating with daughter HCC at the right anterosuperior portal segment. The beam energy and spread-out Bragg peak (15) were fine-tuned so that a 90% isodose volume of the prescribed dose encompassed the PTV.

Forty-six patients received PRT to a total dose of 76 CGE in 3.8 CGE once-daily fractions, four to five fractions in a week. Another 3 patients underwent 60 CGE /10 fractions/2 weeks, depending on availability of the proton beam. Eleven patients whose PTV encompassed the gastrointestinal wall received 65 CGE in 2.5 CGE /fraction, five fractions per week. All patients were treated using a 150- to 190-MV proton beam. The relative biological effectiveness of our proton beam was defined as 1.1 (16). No concomitant treatment such as TACE, local ablation, or systemic therapy was allowed during or after the PRT, unless a treatment failure was detected. Both scanning of CT images for treatment planning and irradiation by the proton beam were done during the exhalation phase using the respiration-gated irradiation system and intrahepatic fiducial markers as previously reported (3).

Outcomes

Death from any cause was defined as an event in calculation of OAS, whereas tumor recurrences at any site or patient deaths were defined as events in disease-free survival (DFS). An increase of the tumor diameter within the PTV was defined as local progression, and patients who died without evidence of local progression were censored at the time of last radiographic examination. Adverse events were reviewed weekly during the PRT regimen by means of physical examination, complete blood count, liver function tests, and other biochemical profiles as indicated. The severity of adverse events was assessed using the National Cancer Institute common terminology criteria for adverse events, version 3.0. After completion of PRT, reviews that monitored disease status, including CT and/or MRI examinations and long-term toxicity, were done at a minimum frequency of every 3 months in all 60 patients. The percentages of hepatic noncancerous portions (entire liver volume minus gross tumor volume) receiving CGE doses of >0 (V0), ≥10 (V10), ≥20 (V20), ≥30 (V30), ≥40 (V40), and ≥50 (V50) were calculated using PRT planning software (PT-PLAN/NDOSE System, Sumitomo Heavy Industries Ltd., Tokyo, Japan), and their influence on the outcomes were analyzed (3). Time-to-event analyses were done using Kaplan-Meier estimates from the start of PRT. The differences between time-to-event curves were evaluated with the log-rank test. Multivariate analyses were performed with Cox's proportional hazards model.

RESULTS

Patients

A total of 60 patients with HCC underwent PRT in our institution between May 1999 and July 2007. Approximately 1400 patients with HCC were newly presented to our institution during this study period and about 35%, 30%, 25%, and the remainder primarily treated with hepatectomy, TACE, percutaneous local ablation, and other treatments, respectively. Therefore 60 patients in this study corresponded to approximately 4% of overall, or 7% of patients with unresectable HCC. Patient characteristics at the start of PRT are listed in Table 1. All patients had underlying chronic liver disease. One patient had a history of schistosomiasis, and another patient had autoimmune hepatitis as the cause of liver cirrhosis. Five additional patients were diagnosed with liver cirrhosis caused by non-B, non-C hepatitis. A total of 24 patients received PRT as the first treatment for their HCC. Ten patients had postsurgical recurrences, 22 patients received unsuccessful local ablation and/or TACE to the targeted tumor, and 4 patients underwent successful local ablation to a tumor other than the target prior to PRT. Histological confirmation was not obtained in 1 patient who had a tumor with typical radiographic features compatible with HCC (3). Six patients had HCC nodules of ≤3 cm in diameter; however, they were not considered candidates for local ablation therapy because of the tumor locations, which were in close proximity to the great vessels or the lung.

Adverse events during PRT

All patients completed the treatment plan. Prolongation of the overall treatment time for more than 1 week occurred in 4 patients: treatment of 3 patients was extended due to availability of the proton beam machine, and 1 patient's treatment was extended because of fever associated with grade 3 elevation of total bilirubin that spontaneously resolved within a week. A total of 14 patients experienced transient grade 3 leukopenia and/or thrombocytopenia without infection or bleeding that necessitated treatment. In addition, 8 patients experiencing grade 3 elevation of transaminases without clinical manifestation of hepatic insufficiency maintained good performance status. PRT was not discontinued for these patients; nevertheless, these events spontaneously resolved within 1 to 2 weeks.

Estimation of the risk of PHI by DVH analysis

Development of hepatic insufficiency presented with anicteric ascites and/or asterixis within 6 months after completion of PRT in the absence of disease progression was defined as PHI. Eleven patients, all of whom received a total PRT dose of 76 CGE, developed PHI at 1 to 6 months (median, 2 months) after completion of PRT without elevation of serum bilirubin and transaminases of more than threefold above normal levels. DVHs for hepatic noncancerous portions were drawn according to pretreatment ICG R15 values (Fig. 1A–C). Results showed that all 20 patients with ICG R15 of <20% were free of PHI, regardless of the DVH, for 2 to 94 months (median, 44 months). On the other hand, 6 of 8 patients with pretreatment ICG R15 values of ≥50% died of PHI with (n = 3) or without (n = 3) evidence of HCC recurrence at 2 to 15 months (median, 8 months). There was no obvious relationship between DVH and development of PHI in these 8 patients, as shown in Fig. 1C.

Among 32 patients whose ICG R15 values ranged from 20% to 49.9%, 5 patients developed PHI. The V0 to V50 in these 32 patients are shown in Fig. 2. Differences in distributions of these DVH parameters between patients who did

Table 1. Characteristics of patients

Characteristics	No. of patients (%)
Age (years)	
Median	70
Range	48–92
Gender	
Male	42 (70)
Female	18 (30)
ECOG performance status	
0–1	57 (95)
2	3 (5)
Viral markers	
Hepatitis B surface antigen-positive	3 (5)
Hepatitis C antibody-positive	49 (82)
Both positive	1 (2)
Both negative	7 (12)
Child-Pugh classification	
A	47 (78)
B	13 (22)
C	0
% patients with pretreatment ICG R15 values	
<20	20 (20)
20–40	25 (55)
40–50	7 (12)
≥50	8 (13)
Tumor size (mm)	
Median	45
Range	20–90
20–50	42 (70)
>50	18 (30)
Macroscopic vascular invasion	
Yes	42 (70)
No	18 (30)
Morphology of primary tumor	
Single nodular	45 (75)
Multinodular, aggregating	9 (15)
Diffuse	5 (8)
Portal vein tumor thrombosis	1 (2)
Serum alpha-fetoprotein level (IU/mL)	
<300	41 (68)
≥300	19 (32)
Histology	
Well-differentiated	15 (25)
Moderately-differentiated	28 (47)
Poorly-differentiated	7 (12)
Differentiation not specified	9 (15)
Negative (radiological diagnosis only)	1 (2)
Prior treatment	
None	24 (40)
Surgery	10 (17)
Local ablation/TACE	26 (43)

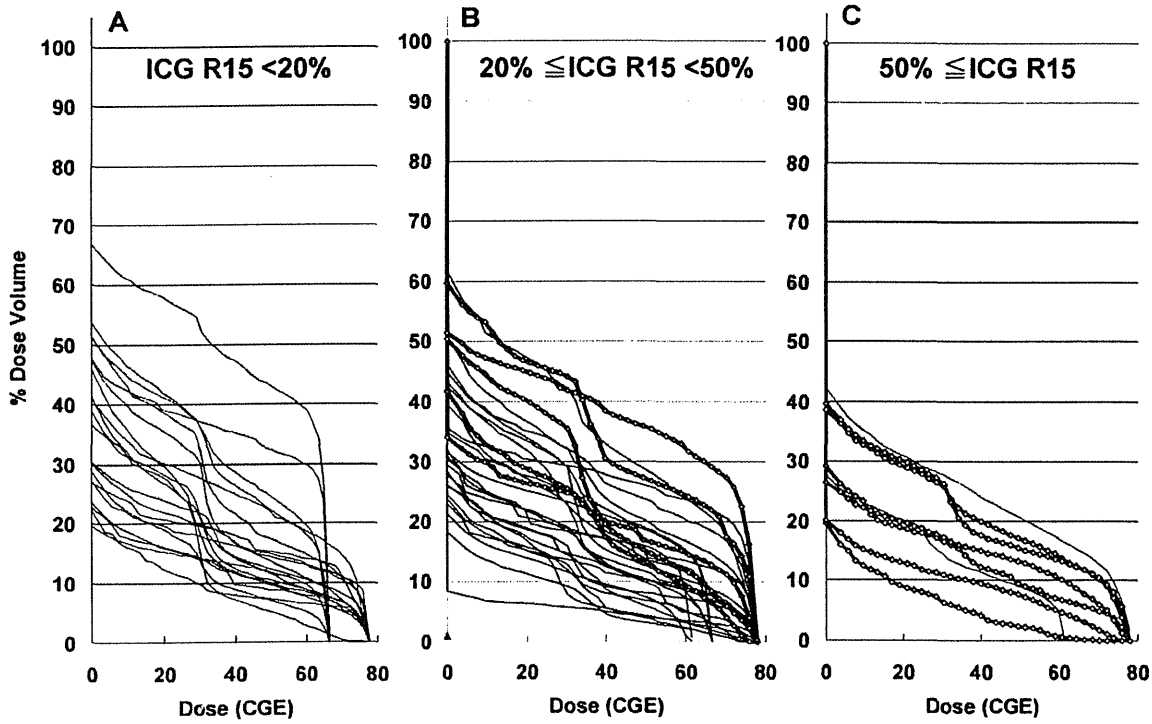

Fig. 1. DVH are shown for all patients according to their pretreatment ICG R15 values, as noted in each panel. Thick lines with rhomboid symbols represent DVHs for patients suffering from hepatic insufficiency within 6 months after completion of PRT.

and did not develop PHI were statistically significant, with p values of 0.012 in V0, 0.009 in V10, 0.012 in V20, 0.006 in V30, 0.016 in V40, and 0.024 in V50 (Mann-Whitney U test). The lowest p value was observed in the difference at V30. Among 32 patients whose ICG R15 values ranged from 20% to 49.9%, none of the 21 patients whose V30 were <25% experienced PHI, whereas 5 of 11 patients (45%) whose V30 was ≥25% developed PHI (p = 0.037, Mann-Whitney U test). The incidence of PHI was 2/25 (8%) in Child-Pugh class A patients, whereas PHI incidence was 3/7 (43%) in class B patients in this group of 32 patients (p = 0.218, Mann-Whitney U test). Of 5 patients who experienced PHI, 1 died at 8 months without evidence of HCC recurrence. PHI spontaneously resolved in 4 patients; 2 patients died of intrahepatic recurrence at 22 and 71 months, respectively; 1 patient died of brain metastasis at 8 months; and 1 patient was alive and disease free at 50 months. In both of the patients who survived for more than 4 years despite development of PHI, the pretreatment functional liver reserve was Child-Pugh class A and ICG R15 was less than 40%. On the other hand, all 3 patients who experienced PHI and died within 2 years had Child-Pugh class B liver functions. Relationships between ICG R15 and V30 according to occurrence of PHI in Child-Pugh class A and B patients are shown in Fig. 3a and b, respectively.

Other serious adverse events

Three patients experienced a gastrointestinal toxicity grade of ≥2. One patient developed hemorrhagic duodenitis associated with anemia at 2 months after completion of 76 CGE/20 fractions/30 days of PRT. The dose administered to the duodenum was estimated to be 50 to 80% of the prescribed dose. Bypass surgery was attempted to alleviate the symptoms; however, this patient died of postoperative hepatic failure at 6 months. Two patients received 65 CGE/26 fractions of PRT, with the entire circumference of the gastrointestinal walls covered within the PTV. One of these 2 patients experienced grade 3 hemorrhagic ulcer at the ascending colon, within the PTV. The patient was managed successfully with right hemicolectomy at 10 months; however, the patient

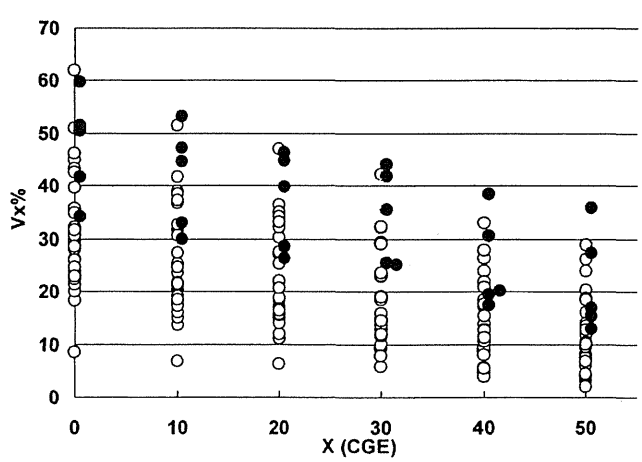

Fig. 2. Distribution of V0 to V50 in DVHs for 32 patients whose pretreatment ICG R15 values ranged from 20% to 49.9%. Open circles represent values for patients who did not experience PHI, whereas closed circles represent those who developed PHI.

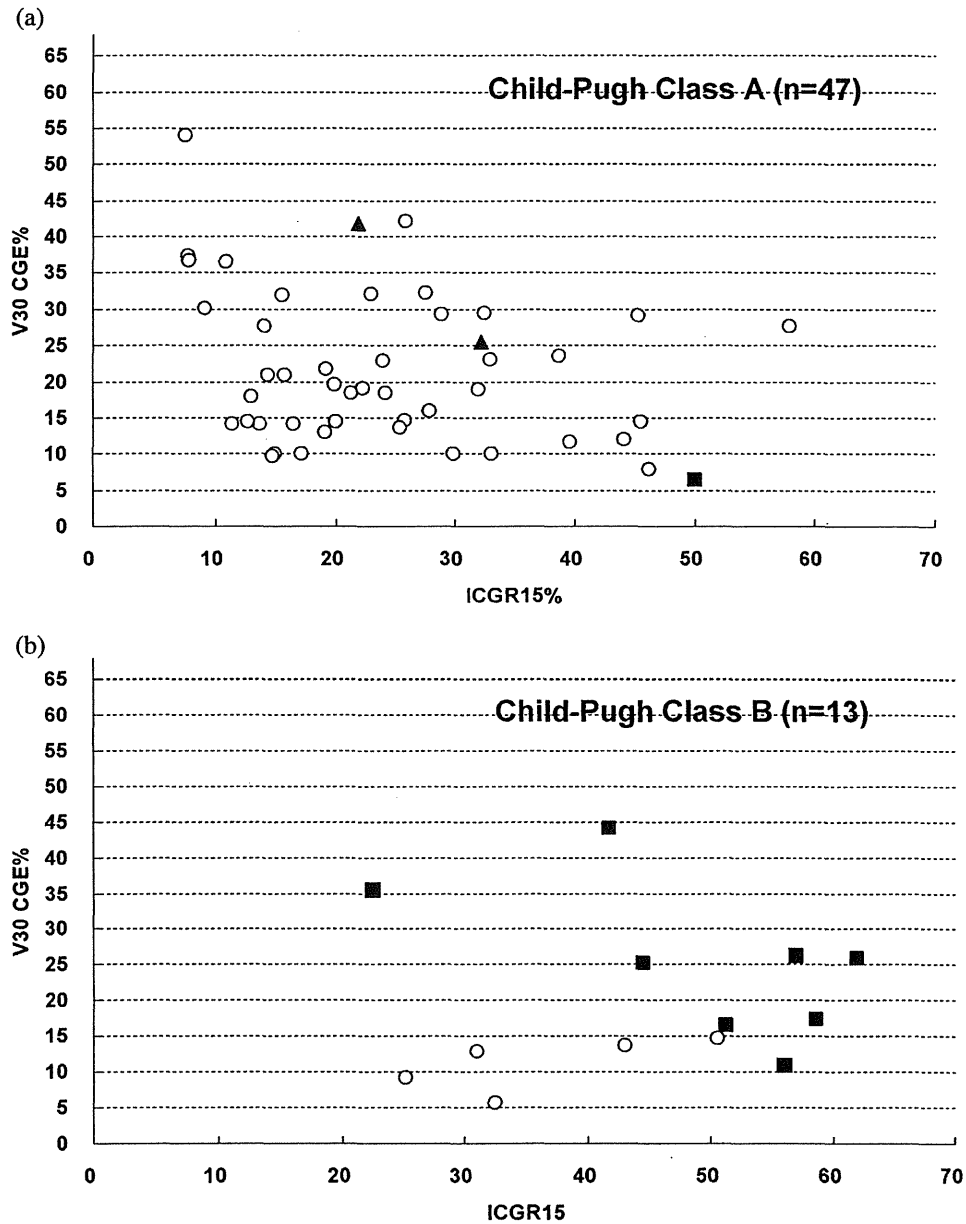

Fig. 3. Scattergram of V30 in each patient who had pretreatment liver functions classified as Child-Pugh class A (a) and class B (b), as shown in each panel, according to the ICG R15 value. Open circles represent values in patients who did not experience PHI. Closed squares represent those who developed PHI and died within 2 years with ($n = 5$) or without ($n = 4$) disease recurrence. Closed triangles represent those who experienced transient PHI and survived for more than 4 years after commencement of PRT.

died of local recurrence and subsequent hepatic failure at 23 months. The other patient developed grade 2 esophagitis within the PTV at 7 months. Repetitive balloon dilatations were required to alleviate the patient's dysphagia; however, the patient was alive without disease and taking a normal diet at 30 months. There were no other observations made of adverse events of Grade ≥3 in any of the patients.

Tumor control and survival

At the time of analysis in August 2009, 42 patients had already died because of intrahepatic recurrence in 27, nodal recurrence in 1, distant metastasis in 3, hepatic insufficiency without recurrence in 9, comorbidity in 1, and senility in 1. Forty of these 42 patients had been free from local progression until death; the durations ranged from 2 to 77 months (median, 20 months). Two patients who experienced local progression died subsequently. A total of 15 patients were alive at 25 to 92 months (median, 43 months) without local progression. Three patients were alive at 49, 53, and 94 months, respectively, after salvage treatment for local progression, using local ablation in 2 and TACE in 1 A total of 37 patients achieved complete disappearance of the primary tumor at 1 to 50 months (median, 10 months) post-PRT. Eighteen patients had residual tumor masses on CT

and/or MRI for 2 to 44 months (median, 21 months) until the time of death or last follow-up visit without local progression. The local progression-free (LPF) rates at 3 and 5 years were 90% (95% confidence interval [CI], 80%–99%) and 86% (95% CI, 74%–98%), respectively.

Of 5 patients who experienced local progression, 3 patients underwent 65 CGE/26 fractions, and 2 patients received 76 CGE/20 fractions of PRT. All 3 patients who received 60 CGE/10 fractions were free from local progression at 6, 30, and 51 months, respectively. LPF rates at 3 and 5 years for 46 patients who received 76 CGE/20 fractions were 97% (95% CI, 92%–100%) and 93% (95% CI, 83%–100%), respectively. LPF rates at 3 years for 11 patients who underwent 65 CGE/26 fractions of PRT were 56% (95% CI, 16%–95%) and was worse than that in patients who received 76 CGE/20 fractions with statistical significance ($p = 0.005$).

A total of 32 patients developed intrahepatic tumor recurrences that were outside of the PTV at 1 to 62 months (median, 20 months). Nine of these tumors occurred within the same segment of the primary tumor. Nodal recurrence at the hepatoduodenal ligament and distant metastasis were observed as the first sites of failure in 2 and 3 patients, respectively. In addition to the above-mentioned five deaths from PHI or postsurgical mortality, 4 patients died of hepatic failure because of underlying liver disease at 17 to 23 months, and 2 patients died from other reasons (comorbidity or senility) without evidence of HCC recurrence. Seven patients remained alive and disease free at 27 to 51 months (median, 30 months). The median survival time for all 60 patients was 41 months, and actuarial OAS rates at 3 and 5 years were 56% (95% CI, 43%–69%) and 25% (12%–39%), respectively. DFS rates at 3 and 5 years were 18% (95% CI, 7%–29%) and 4% (95% CI, 0%–12%), respectively, as shown in Fig. 4. Two Child-Pugh class A patients who underwent PRT with the CTV covering the entire right lobe or right anterior portal segment were alive and disease free at 50 and 26 months, respectively. The former patient had a pre-PRT ICG R15 of 22% and received a V30 of 42% and experienced transient PHI that resolved spontaneously; the latter patient, whose corresponding parameters were 8% and 37%, respectively, did not experience PHI.

Factor analysis

Univariate analyses revealed that factors related to functional liver reserve and occurrence of PHI had significant influence on OAS ($p < 0.05$). Liver function (Child-Pugh class A or B) and prior treatment (none or recurrent) were independent and significant prognostic factors ($p < 0.002$), and occurrence of PHI had marginal significance ($p = 0.011$) by multivariate analysis, as shown in Table 2. The DFS rate at 3 years for 24 patients who had no prior treatment for HCC was 35% (95% CI, 14%–56%), whereas DFS for the remaining 36 patients was 7% (95% CI, 0%–17%) ($p = 0.011$). In Child-Pugh class A patients, OAS at 3 and 5 years for those who had no prior treatment ($n = 17$) was 76% (95% CI, 56%–97%) and 59% (95% CI, 33%–86%), respectively, and 63% (95% CI, 45%–80%) and 25% (95% CI, 7%–42%), respectively, for 30 patients with recurrent tumor ($p = 0.060$). In Child-Pugh class B patients, the 2-year OAS for patients without PHI ($n = 5$) was 80% (95% CI, 45%–100%), while 8 patients who developed PHI died within 2 years with ($n = 5$) or without ($n = 3$) HCC recurrence ($p = 0.009$).

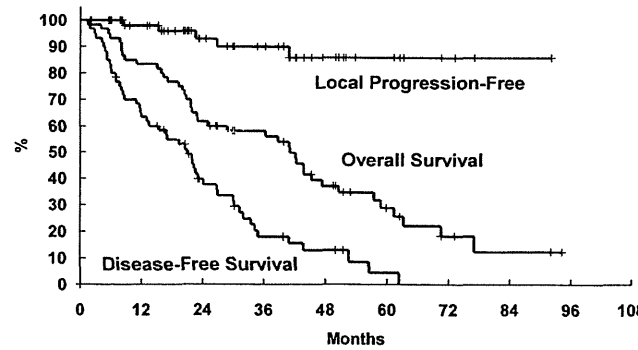

Fig. 4. Kaplan-Meier estimation of local progression-free survival, OAS, and disease-free survival rates for all 60 patients.

DISCUSSION

The promising tumoricidal effect of PRT using aggressive escalation of total and fractional doses, which has been repeatedly reported previously, was reproduced in this study (3, 4). The estimated actuarial local progression-free rate within the PTV in patients receiving 76 CGE/20 fractions exceeded 90% at 3 years. DFS at 3 years for patients who underwent PRT as an initial treatment ($n = 24$) was 35%, and, among them, OAS at 3 years was 76% in Child-Pugh class A patients ($n = 17$). These results are comparable to those observed after surgical treatment (17). Although the number of patients was small, these data indicate that appropriate local control with PRT may provide survival benefit in adequately selected patients with unresectable HCC. The fact that 9 of the 32 intrahepatic HCC recurrences occurred within the same anatomical portal segments showed that it should still be possible to improve the progression-free rate by defining the CTV so it covers undetectable tumor spread via the portal blood flow.

As shown in Fig. 3, no patient who had ICG R15 of less than 20% experienced PHI. In addition, only Child-Pugh class A patients with pre-PRT ICG R15 of less than 40% survived for longer than 4 years despite development of PHI. One of them underwent systematic portal segmental irradiation with the CTV covering the entire right lobe, and the details for this patient will be reported separately. On the other hand, all patients who had pre-PRT liver functions classified as Child-Pugh class B and/or ICG R15 of 40% or higher died within 2 years when they developed PHI. This suggests that the role of systematic portal irradiation requiring a large irradiated volume should be pursued further in Child-Pugh class A patients with favorable ICG R15 values; otherwise, the CTV should be confined to the GTV with adequate margins. Furthermore, in patients who have ICG R15 of 50% or