Alterations in DNA damage repair genes in primary liver cancer.

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Translational Relevance

The present study investigated the frequency and translational significance of DNA damage repair (DDR) genes in primary liver cancer (PLC). Utilizing targeted deep sequencing of all exons and selected introns of 450 key cancer-related genes in a total of 357 PLC patients, we found that 25.8% of patients carried at least one mutation in DDR genes, 15 of whom carried germline mutations. Comparative analysis indicates that patients with DDR mutations have significantly higher tumor mutation burden. Among the patients with DDR mutations, 26.1% (24/92) of patients possessed at least one actionable alteration, and the actionable frequency in DDR wild type PLC was 18.9% (50/265). Eight patients with advanced ICC were treated with olaparib, and we found that patients with BRCA truncation germline mutations tended to obtain an objective response. These findings suggest that identifying DDR mutated PLC can facilitate and broaden the clinical application of precision oncology and that specific genotypes can inform therapeutic implications and outcomes in terms of targeted treatment and immunotherapy.
Abstract

Purpose: Alterations in DNA damage repair (DDR) genes produce therapeutic biomarkers. However, the characteristics and significance of DDR alterations remain undefined in primary liver cancer (PLC).

Experimental Design: Patients diagnosed with PLC were enrolled in the trial (PTHBC, NCT02715089). Tumors and matched blood samples from participants were collected for a targeted next-generation sequencing assay containing exons of 450 cancer-related genes, including 31 DDR genes. The OncoKB knowledge database was used to identify and classify actionable alterations, and therapeutic regimens were determined after discussion by a multidisciplinary tumor board.

Results: A total of 357 PLC patients were enrolled, including 214 with hepatocellular carcinoma, 122 with intrahepatic cholangiocarcinoma (ICC) and 21 with mixed hepatocellular-cholangiocarcinoma. A total of 92 (25.8%) patients had at least one DDR gene mutation, 15 of whom carried germline mutations. The most commonly altered DDR genes were ATM (5%) and BRCA1/2 (4.8%). The occurrence of DDR mutations was significantly correlated with a higher tumor mutation burden regardless of the PLC pathological subtype. For DDR-mutated PLC, 26.1% (24/92) of patients possessed at least one actionable alteration, and the actionable frequency in DDR wild type PLC was 18.9% (50/265). Eight BRCA-mutated patients were treated by olaparib, and patients with BRCA2 germline truncation mutations showed an objective response.

Conclusions: The landscape of DDR mutations and their association with genetic and clinicopathological features demonstrated that PLC patients with altered DDR genes may be rational candidates for precision oncology treatment.
Introduction

Primary liver cancer (PLC) is the fifth leading cause of cancer deaths (1), and is more prevailing in East Asia and Western Europe (2). Globally, major pathological types of PLC include hepatocellular carcinoma (HCC), intrahepatic cholangiocarcinoma (ICC) and hepatocellular-cholangiocarcinoma (H-ChC). HCC is the most common subtype of PLC, accounting for approximately 80% of total cases. ICC and H-ChC are uncommon subclasses of PLC and have poorer prognosis and shorter overall survival than HCC does (3). The etiology of PLC highlights the main risk factors, including hepatitis virus infections (HBV or HCV), gender (male), individual behaviors (alcohol or smoking), metabolic disorders (diabetes or obesity) and aflatoxins (4,5).

Advancements in genomic sequencing have facilitated the elucidation of the PLC mutational landscape, characteristics and signatures. Integrative genomic multiomics analysis has revealed varied mutational features of PLC across pathological types and risk factors (6), suggesting that PLC has complex genomic alterations with a high level of heterogeneity and instability in the cancer genome (7,8). PLC with hepatitis virus infections is associated with DNA damage (9,10). Responses to DNA damage mainly rely on enzymes encoded by DNA damage repair (DDR) pathways. Seven functional gene sets are involved in DDR pathways: homologous recombination (HR), mismatch repair (MMR), base excision repair (BER), nucleotide excision repair (NER), nonhomologous end-joining (NHEJ), checkpoint factors (CPF) and Fanconi anemia (FA) (11,12). Accumulating evidences indicate that dysfunctions or defects in DDR genes are related to cancer susceptibility and occurrence for some sporadic cancers, including breast, ovarian, urothelial and pancreatic cancers. However, the mutational spectrum of DDR pathways and the significance in PLC remain to be unelucidated.

Importantly, the role of DDR mutations in cancer has attracted increasing attention because of their cancer-driving effects and significance in clinical and translational medicine, which could broaden therapy options for patients with advanced PLC. For example, cancer patients carrying the BRCA1/2 mutation are suitable for poly-ADP-ribose polymerase inhibitor (PAPRi) treatment (13,14). DDR alterations are positively correlated with a higher tumor mutation burden (TMB) (15) and are independently associated with the
therapeutic response to PD-1/PD-L1 inhibitors(16). Moreover, many studies have demonstrated that overexpression of DDR pathway molecules confers intrinsic resistance to cisplatin(17) while tumors with deleterious DDR mutations are more sensitive to platinum-based therapy(18).

To elucidate the significant but undefined role of DDR mutations in PLC, in the present study, we investigated the DDR mutational landscape and its translational meaning in clinical precision treatment for PLC patients, which was based on the results from our registered trial termed "Precision Treatment for Hepatobiliary Cancer" (PTHBC, NCT02715089).

Methods and materials

Patients and study population

Patients with PLCs, including pathologically confirmed HCC, ICC and mixed H-ChC, were eligible for our study (PTHBC, NCT02715089). Informed consent was obtained for tumor profiling and targeted therapy following protocol approved by the Institutional Ethics Review Committee at Peking Union Medical College Hospital (PUMCH). The study was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines. All patients signed consent before participating in the research.

Sample collection and preparation

Tumor samples were obtained from participants at different clinical stages. Detailed information of the samples is summarized in Table 1. All tumor tissues were reviewed by two independent pathologists before sample disposal to confirm the pathological diagnoses. Macrodissection on tissue slides was performed to evaluate tumor content and percentage. Only samples with estimated tumor purity >20% on histopathological assessment were further subjected to genomic profiling. Peripheral blood was collected from each patient as the normal control sample for genomic profiling.
Targeted next-generation sequencing (tNGS) and genetic analysis

Genomic profiling was performed in the laboratory of OrigiMed (Shanghai, China). At least 50 ng of cancer tissue DNA was extracted from each 40-mm FFPE tumor sample using a DNA Extraction Kit (QiAamp DNA FFPE Tissue Kit) according to the manufacturer’s protocols. All coding exons of 450 key cancer-related genes and selected introns of 36 genes commonly rearranged in solid tumors were incorporated into the custom hybridization capture panel. In addition, the probe density was increased to ensure high efficiency of capture in the conservatively low read depth region. Libraries were each diluted to 1.05 nM and then sequenced with a mean coverage of 900X for FFPE samples and 300X for matched blood samples on an Illumina NextSeq-500 Platform (Illumina Incorporated, San Diego, CA).

Genomic alterations, including single nucleotide variations (SNVs), short and long insertions/deletions (indels), copy number variations (CNVs), gene rearrangements and gene fusions, were subjected to advanced analysis. First, reads were aligned to the human genome reference sequence (hg19) by Burrows-Wheeler Aligner (BWA), and PCR duplicates were removed using Picard. Second, SNVs and short indels were identified by MUTECT after quality recalibration and realignment using GATK. Short indels were then calibrated using the results from Pindel. Moreover, read depths were normalized within target regions by EXCATOR. The log-ratio per region of each gene was calculated, and customized algorithms were used to detect CNVs. Germline variants were identified by HaplotypeCaller from the Genome Analysis Toolkit (GATK v.3/3) in the gvcf mode with default settings(19), and only those present in both normal and tumor samples were retained. Tumor cellularity was estimated by allele frequencies of sequenced SNPs. Third, a customized algorithm was developed to detect gene rearrangements, fusions and long indels. TMB was estimated following the methods of Chalmers et al(20). Briefly, the total numbers of somatic, coding, base substitutions and short indels were counted; driver mutations and known germline alterations in dbSNP were not counted. Then, TMB was calculated by dividing the total number of mutations counted by the size of
the coding region. We used 1.25 megabases (Mb) as the coding region size of the YuanSu™ panel.

Reliable somatic alterations were detected in the raw data by comparison with matched blood control samples. At minimum, 5 reads were required to support alternative calling. For CNVs, focal amplifications were characterized as genes with thresholds ≥4 copies for amplification and 0 copies for homozygous deletions. Clinically relevant genomic alterations were further marked as druggable genomic alterations in current treatments or clinical trials.

All alterations for each patient in our cohort were compiled and summarized in Supplementary Table S2.

**Annotation for mutations of DDR genes**

The functional significance of variants in DDR genes was determined by interrogating databases and published literature, such as ClinVar, Catalogue of Somatic Mutations in Cancer (COSMIC), and PubMed. Known or likely drivers and recurrent variants were reported in our study, pathogenic mutations were defined as those variants that would clearly have an effect on the function of a gene, including nonsense, frameshift, start/stop codon changes, and splice site mutations. The evidence for pathogenic variations mainly derived from the public databases, including the Human Gene Mutation Database (HGMD), Clinvar, Sorting Intolerant From Tolerant (SIFT), and the standard from American College of Medical Genetics (ACMG).

**Identification and classification of actionable alterations**

The actionabilities of genetic alterations were referred to as the OncoKB knowledge database, which comprehensively considered the guidelines and recommendations from the FDA, NCCN and medical literature(21). All actionable alterations were classified as level 1, 2A/B, 3A/B and 4. According to the annotations of OncoKB, level1 alterations include genes whose alterations were recognized by the FDA as predictive of response to an FDA-approved drug in a specific cancer type, such as vemurafenib or dabrafenib in BRAF^{V600E} melanoma, and a total of 82 alterations from 12 genes were determined as
level 1. Level 2 consists of parts A and B parts. Level 2A includes alterations that are considered standard care predictive biomarkers of response to an FDA-approved therapy in some particular cancer types but have not been recognized by the FDA, which were recommended by NCCN and ASCO clinical practice guidelines within the indications. For example, using olaparib in breast cancer patients with oncogenic mutations of BRCA2. If the predictive biomarkers of response to an FDA-approved drug are not recommended by guidelines, which the indications are out of standard care, these are classified as level 2B. An example is using olaparib in cholangiocarcinoma patients with oncogenic mutations of BRCA2. Level 3 also has two sublevels. Level 3A includes mutations with compelling clinical evidence in reported tumor types, which are regarded as the biomarkers of therapeutic response for off-label use of FDA-approved drugs or investigational agents that are not yet approved by the FDA. If the tumor types have not been reported, then the level is classified as level 3B. Level 4 alterations are candidate predictive biomarkers of response to targeted agents on the basis of compelling laboratory data with biological evidence.

In our study, we deemed that alterations between level 1 and level 3A were actionable targets, meaning that targeted therapeutic regimens based on actionable alterations were discussed by a multidisciplinary tumor board. For mutations within level 1-4, we defined all these alterations as translational targets.

For actionable alterations, the levels of evidence for the corresponding drugs have been respectively annotated in three different databases, including OncoKB, DGIdb (v3.0.2)(22) and PanDrugs (version: 2018.11.7)(23) (Supplementary Table S4).

Treatments

For patients who were identified as carriers of actionable targets, therapeutic targeted drugs were administered according to the genetic test reports. Once the patients received targeted treatments, follow-up was conducted to evaluate the efficacy and safety of the drugs until the determination of overall survival.

Eligible patients to receive therapeutic target drugs must have at least one actionable alteration, who required palliative care after at least two failures of anti-tumor therapies.
Previous adjuvant treatment with platinum was allowed if at least 3 months had elapsed since the last dose. Patients were required to have 0-2 Eastern Cooperative Oncology Group (ECOG) status and normal baseline organ and bone marrow function. All patients to receive targeted drugs had at least one measurable lesion that was used for assessing the therapeutic response according to the criteria of Response Evaluation Criteria in Solid Tumors (RECIST), version 1.1(24).

In the present trial, for BRCA mutated patients with liver cancer who received targeted treatment, the therapeutic drug was olaparib. The initial dosage was 200mg twice a day. For patients who were intolerant to this dosage, 100mg twice a day or treatment interruption was available. Computed tomography or magnetic resonance imaging was performed every 6-8 weeks to determine the therapeutic response. Adverse events were graded through Common Terminology Criteria for Adverse Events (CTCAE), version 4.0.

**Statistical analysis**

All statistical analyses were performed using R version 3.4.2. Continuous variables are expressed as the mean ± standard deviation if they were normally distributed, otherwise as the median with interquartile ranges are presented. The R package “PMCMRplus” was used to perform the Kruskal-Wallis rank sum test and Anderson-Darling all-pairs comparison test for nonnormally distributed continuous data. The R package “rcompanion” was used to conduct Fisher’s exact test or Chi-squared test and post hoc tests for comparisons of multiple frequencies. Linear models were fitted by the R function “lm”. Variables with a value less than 0.05 on univariate linear regression were included in the multivariable linear regression model. The R packages “ggplot2” and “ComplexHeatmap” were used to draw figures. All reported p-values were two-tailed, and p < 0.05 was considered statistically significant.

**Results**

**Characteristics of the study population**
In this study, tumor tissues and paired blood samples were obtained from a total of 357 patients with pathologically confirmed PLC, including 214 (60%) with HCC, 122 (34%) with ICC and 21 (6%) with H-ChC. Briefly, 78.7% (281/357) were male, and the median age for our study population was 56 (range: 16 - 88) years. A total of 44.5% of patients possessed a confirmed cancer-related family history. The characteristics of the study population are summarized in Table 1. For tissue origins, 90.8% were obtained from primary tumors, while 9.2% were obtained from metastasis sites. A total of 83.2% of samples were obtained before systemic chemotherapy or transcatheter arterial chemoembolization (chemotherapy-naive). 86.8% of the tissues were obtained from surgical resection, and 13.2% were obtained from regional needle biopsy.

**Landscape of DDR mutations in PLC**

To depict the landscape of DDR mutations in PLC, we used a tNGS panel that captured mutations in coding regions of 450 cancer-related genes, including 31 DDR genes and partial intron regions of 36 genes (Supplementary Table S1). These DDR genes covered by the panel are known cancer susceptibility genes and were mutated in PLC according to previous reports(25). As most DDR genes have not yet been determined to have oncogenic effects, we reported 31 DDR gene mutations that were available in published literature and public variant databases, such as the Catalogue of Somatic Mutations in Cancer (COSMIC)(26) and OncoKB(21).

92 of 357 (25.8%) patients had at least one mutation in DDR genes, including 49 of the HCC patients, 37 of the ICC patients and 6 of the H-ChC patients (Table 2). The most common mutational type was substitutions/indels (54.24%), followed by truncation (36.44%, Figure 1A). The most frequently mutated individual DDR genes included ATM (5%), BRCA1/2 (4.8%). For different pathological subtypes of PLC, the frequencies and distributions of DDR mutations varied. A total of 6.07% of HCC patients had mutated ATM, while ICC patients possessed a high burden of BRCA1/2 mutations (9.02%). Alterations in ATR, APEX1 and MUTYH were only identified in HCC patients. Mutations in POLE and POLD1, which can cause genetic instability and cancer mutation accumulation, occurred in 5 HCC patients and 1 ICC patient (Figure 1B). Among the 6 functional categories of
DDR genes, we found that mutations of checkpoint factor (CPF) were enriched in HCC, while alterations in homologous recombination repair (HRR) were more common in ICC. We also compiled the spectrum of DDR mutations in 92 DDR\textsuperscript{mut} PLC patients (Supplementary Figure S1).

Germline DDR mutations are found primarily in breast and ovarian cancers and sporadically occur in pancreaticobiliary cancers. For its vague role in PLC, we next investigated germline DDR deficiency in these 357 patients. As a result, a total of 15 patients (7 with HCC and 8 with ICC) had deleterious germline mutations in BRCA2, BRCA1, ATM, PMS2, BLM, FANCA, MLH1 and RAD50 (Figure 1C). We further verified these germline variants and confirmed that, except for one case that was a missense substitution of MLH1, the remaining variations were truncated in the coding regions. Intriguingly, all 4 patients with BRCA2 germline deleterious mutations were diagnosed with ICC, which was consistent with previous reports that carriers of germline mutations in BRCA2 are at high risk for bile tract cancer and pancreatic cancer(27). A deleterious mutation in the germline may indicate family heredity, so we processed a family study for 77 DDR\textsuperscript{mut} PLC patients, excluding 15 DDR\textsuperscript{mut} participants who were unwilling to provide family cancer history. Overall, 33.77% (26/77) of patients had a family history of cancer, and the majority of family members with cancers were diagnosed with PLC. We further screened 10 of 15 patients who were identified as having germline DDR mutations and found that only 3 carriers with germline mutations in BRCA2 had susceptible genetic hereditary phenomena in their families (Figure 1D-F).

**Mutations in DDR genes, especially in BER/FA/MMR, indicate higher TMB**

Alterations in DDR genes interfere with the capability of repairing different sets of DNA lesions, inducing those that confer genetic and chromosomal instability(28). This mechanism results in cancer with DDR mutations accumulating extensive genomic mutations, which leads to an elevated TMB. Whether this phenomenon exists in PLC has not yet been determined. Here, we investigated the correlation between DDR\textsuperscript{mut} PLC and TMB levels.
The median (quantile) TMB for the study population of 357 PLC patients was 4.0 (2.3-7.8) mutations/Mb (Mut/Mb). First, we demonstrated that TMB in HCC patients was significantly higher than that in ICC patients (p=0.043, Figure 2A), which was consistent with results from the TCGA(6,29). Then, we confirmed that patients with DDR mutations had a significantly higher TMB than did patients with wild type DDR genes (p<0.001, Figure 2B), as the same in the different pathological types (all p<0.05, Supplementary Figure S2A). Furthermore, using the upper-quantile value (≥7.8 Mut/Mb) to identify the patients with high TMB, DDR\textsuperscript{mut} PLC had a significantly higher rate of TMB-high patients than DDR\textsuperscript{mut} PLC (41.3% vs 20.0%, p<0.001). Moreover, among the DDR\textsuperscript{mut} PLC subgroup (N=92), DDR\textsuperscript{mut} HCC had significantly higher TMB than did DDR\textsuperscript{wt} ICC (p=0.043, Figure 2C). To validate the positive correlation between DDR mutations and TMB, we also analyzed the TCGA-LIHC cohort of 373 patients diagnosed with HCC. DDR\textsuperscript{mut} patients were defined as those with any nonsilent mutations in DDR genes, and TMB was defined as the number of nonsilent mutations as previously reported(6). Consistent with our study results, patients with DDR mutations had significantly elevated TMB (p<0.001, Supplementary Figure S2B) and greater TMB-high patient rates (49.4%, 43/87 vs 18.5%, 53/286, p<0.001).

To further disclose the main contributing components affecting the correlations between DDR mutations and elevated TMB, we integrated possible confounding factors, including age, sex, pathological differentiation, pathological subtypes, HBV infections, DDR mutations and mutations among the six categories of DDR genes, to conduct a correlation analysis. We found that older age, male gender and DDR mutations were positively related to TMB. Importantly, the mutations in “BFM” (BER/FA/MMR), but not HRR/CPF/NHEJ mutations, were significantly correlated with TMB (p<0.001, Figure 2D). For the DDR\textsuperscript{mut} PLC patients, the BFM\textsuperscript{mut} subgroup also showed a significantly increasing TMB level (p=0.042, Figure 2E).

Overall, these outcomes demonstrated that DDR mutations, especially for genes in BFM, were significantly positively correlated with higher TMB in PLC.

Targeted therapeutic response of BRCAness in PLC
BRCAness represents a subgroup of sporadically occurring tumors with HRR defects(30). For BRCAness, especially for patients with BRCA1/2 pathogenic mutations, a PARPi such as olaparib may possess potent anti-tumor efficacy through a synthetic lethal approach(31). As mentioned above, in our study population, 4.8% (17/357) of patients (5 HCC, 11 ICC and 1 H-ChC) were identified as carriers of BRCA1/2 mutations, who were also matched to targeted therapy with a PARPi. Among the patients with BRCA1/2 mutations, 7 patients exhibited germline mutations, and most (5/7) cases were ICC. There were 6 patients with BRCA fusion, and all these fusion events occurred in somatic tumor cells, with 5 patients with altered BRCA1 and one with BRCA2-FRY rearrangement. Referring to the standards of OncoKB, level 2B actionable patterns of BRCA1/2 mutations include oncogenic mutations and fusions. In our study population, we identified 10 cases of BRCA1/2 oncogenic mutations and 3 cases with BRCA1/2 oncogenic fusions.

Previous studies suggested that both somatic and germline mutations of BRCA1/2 in breast and ovarian cancer could be therapeutically targeted by synthetic lethal efficacy, and thus these cancers were sensitive to PARPi(32,33). However, limited literature has focused on the anti-cancer effect of PARPi compounds in PLC. Herein, we explored 8 BRCAness patients with 7 ICC and 1 H-ChC, who were all treated with olaparib (a PARPi) after several treatment failures. Three patients with germline mutations had a confirmed cancer-related family history with a BRCA oncogenic mutation predisposition, as mentioned above (Figure 1D-F). Therapeutic response and efficacy were different from person to person (Figure 3A-B), 3 patients achieved partial response (PR), 2 patients achieved stable disease (SD) for 3-5 months and 3 patients had progressive disease (PD) at the best response. Intriguingly, all 3 PR patients had germline BRCA2 mutations and family cancer history, highlighting that ICC patients with BRCA2 germline mutations may be more sensitive to PARPi therapy. The detailed locations for the altered amino acids of olaparib-treated patients are presented in Figure 3A. We found that the 3 patients with PR therapeutic efficacy all had truncation mutations of BRCA2, while the 3 PD patients without clinical benefits only carried somatic missense mutations. Considering that synthetic lethality induced by a PARPi requires dysfunction or loss-of-function of
homologous recombination, our results indicate that mutational patterns of BRCA1/2 should be fully evaluated when choosing PARPi treatment in BRCAness PLC patients.

Recent basic and clinical studies have underlined that cancer patients with DDR mutations were more likely to achieve a therapeutic response when receiving immune-checkpoint inhibitors (ICIs)\(^{(16,34)}\). Assumption of combinational therapy of a PARPi plus an ICI has been cited in clinical practice\(^{(35)}\). In our cohort, Patient051 achieved PR for 6 months under olaparib treatment (200 mg twice daily), and after progression, he received olaparib plus pembrolizumab (olaparib 100 mg twice daily + pembrolizumab 140 mg/3 weeks). Although he did not achieve an objective response again, olaparib plus the ICI achieved another 8 months of stable disease without distant metastasis.

**Optional and rational therapeutic targets for DDR\(^{\text{mut}}\) PLC**

To better define the prevalence and cooccurrence patterns of other potentially actionable targets among DDR\(^{\text{mut}}\) PLC, we analyzed and annotated alterations in all enrolled PLC patients (Figure 4A and Supplementary Figure S3). For patients with DDR\(^{\text{mut}}\) PLC, translational pathways mainly included genes related to the with DDR, cell cycle, chromatin-modifying and RTK-PIK3 pathways. The most frequently altered genes were TP53 (46.7%), TERT (27.2%), ATM (19.6%), ARID1A (13.0%) and CTNNB1 (10.9%). Alterations in chromatin-modifying genes, including ARID1A/1B, KMT2C/2D, BAP1 and PBRM1, occurred in 28.3% (26/92) of patients (Figure 4A). We further explored the underlying cooccurring mutations in DDR\(^{\text{mut}}\) PLC and found some cooccurring intendancies with statistically significance in mutations of FGF14/IRS2/TNFSF13B/STK24, while TP53/ATM showed slightly exclusive mutations (Figure 4B). To further investigate the co-occurring mutations in DDR\(^{\text{mut}}\) and DDR\(^{\text{wt}}\) PLC. We firstly selected intersectional mutations of genes among subgroups of DDR\(^{\text{mut}}\) and DDR\(^{\text{wt}}\) patients. Then, we chose above genes with over 5% mutated frequency in all patients (n=357), so that 18 genes were identified as co-occurring mutations for both DDR\(^{\text{mut}}\) and DDR\(^{\text{wt}}\) patients (Supplementary Table S3). We found that the most common co-mutated genes were TP53, TERT, CTNNB1 and ARID1A.
We next defined the frequency of actionable alterations for PLC patients. As there were no standard-of-care targeted agents based on mutations for PLC, no patients had an OncoKB level 1 or 2A alteration to match the targeted therapy. Overall, 51% (182/357) PLC patients (Figure 4C), including 55 DDR$^{\text{mut}}$ PLC and 127 DDR$^{\text{wt}}$ PLC, had at least one translational target which was defined as a nonsynonymous mutation with any level of OncoKB recommendations (21). However, only 26.1% (24/92) DDR$^{\text{mut}}$ PLC patients and 39.4% (50/127) of patients with DDR$^{\text{wt}}$ PLC were identified with actionable targets which include OncoKB recommendations with level 2B or 3A. For 24 DDR$^{\text{mut}}$ patients carried with actionable alterations, 21 patients had alterations that was classified as level 2B and 3 patients possessed only level 3A mutations. Except for BRCA1/2 oncogenic mutations and fusions (13 cases), other actionable alterations include MET amplifications, TSC 1/2 oncogenic mutations, IDH1/2 oncogenic mutations, ERBB2 amplification and FGFR2 fusion (Figure 4D-4E).

For 265 DDR$^{\text{wt}}$ cases, 47.9% (127/265) of patients with DDR$^{\text{wt}}$ PLC had at least one translational target, of whom 18.9% (50/265) of patients carried actionable alterations. Compared with DDR$^{\text{mut}}$ patients, DDR$^{\text{wt}}$ PLC patients had a higher rate of actionable alterations in IDH1/2 and TSC1/2 (Figure 4E). For all translational targets, the matched drugs and its levels of evidence were annotated in three independent databases (Supplementary Table S4), including OncoKB, DGIdb (22) and PanDrugs (23).

**Discussion**

Robust functions of DDR are regarded as the foundation of regular replication and metabolism for cells. The dysfunctions of DDR genes are strongly associated with genomic instability and the accumulation of mutations, favoring cell duplication in the background of excessive DNA base mismatches and chromosomal abnormalities (13). Cancers with frequent DDR mutations, including ovarian cancer, breast cancer and urothelial tumors, tend to have an inclination of family cancer aggregation and are hereditary (36). These phenomena account for the cancer-driving potency of DDR.
mutations. However, the mutational spectra and characteristics of DDR genes in primary liver cancers remain elusive. Relevant factors, such as the genome of HBV, integrate into DDR genes and monitor the role of DDR genes in the process of liver cell regeneration(37), suggesting an underlying correlation between DDR mutations and liver cancers. Moreover, ICC, featured as a bile tract tumor, carries tumor susceptibility when DDR genes exhibit oncogenic mutations(38). Herein, through our study cohort of 357 PLC patients, we disclosed the mutational distribution and variant frequency of DDR genes in patients with PLC. We investigated the relationships between DDR mutations and different pathological types of PLC. Using TCGA-LIHC as a validation cohort, we uncovered a significantly positive association of TMB in PLC patients with DDR mutations. The present study provides a reference for exploring precision oncology in DDR mut PLC patients.

Through deeply targeted genome next-generation sequencing, we found that 25.8% of PLC patients had at least one DDR mutation, which was relatively frequent among HCC patients. In the diverse functional categories and pathways of DDR genes, base excision repair (BER) was the most commonly altered DDR pathway in PLC. The dysregulation of BER function facilitates the accumulation of genomic mutations in cancer cells and benefits tumor subclones to adapt to changes in the tumor microenvironment(39,40). In addition, we discovered a significant yield of deleterious germline mutations in DDR genes in PLC, especially in BRCA1/2 and ATM. A total of 16.3% (15/92) of DDR mut PLC patients had mutations in germline cells, and 33.77% of DDR mut PLC patients had a family cancers history. Family history remains one of the best predictors of future cancer risk, especially for breast, colorectal and ovarian cancers(41), so we further identified 3 independent genealogies with confirmed cancer-susceptible DDR mutation inheritances. Our study highlights the importance and essentiality of risk assessment and primary prevention by using gene testing and genetic counseling for DDR mut PLC patients with family cancer history. Certainly, we should hold rigorous attitudes in concluding that families with DDR mut PLC patients possess higher cancer risk because factors including HBV/HCV spread in family members and aflatoxin contamination in living environments also cause a high incidence of liver cancer.
As mentioned above, DDR mutations accompany aggregating somatic mutations and DNA mismatches, so tumors with DDR mutations are inclined to have increased TMB. In our present study, we demonstrate that DDRmut PLC patients have a significantly higher TMB, which was consistent with previously reported studies in other solid tumors. Importantly, we identified three functional pathways termed “BFM” (BER+FA+MMR) that showed better association with TMB level. In general, higher TMB has associated with poorer survival prognosis, bringing an interesting topic that significantly elevated TMB exists in HCC patients, while the degree of malignancy and survival are poorer in ICC than in HCC. Clinically, there are more effective treatments for HCC, such as transarterial chemoembolization (TACE) and molecular targeted agents, including sorafenib and lenvatinib, which contributes to the improved survival of HCC patients compared with ICC patients. Besides, for the correlation between TMB and survival prognosis, various confounding factors should be comprehensively considered, such as gender, age, smoking habit and disease etiology. In our cohort, compared with ICC, the HCC group had more male patients, a higher rate of HBV/HCV infections (Table 1). These factors, particularly HBV infection, may be a plausible explanation for the higher level of TMB in HCC patients. From the view of genomics, the underlying hypothesis is that HBV-related HCC tends to lack leading oncogenic drivers so that accumulating alterations are required for carcinogenesis and its progression, but ICC possesses more specific drivers such as IDH1/2 mutation, and BRCA mutation. Moreover, HBV infection was a positive factor for better prognosis in ICC patients(42), and antiviral therapy could improve survivals for HBV infected ICC patients(43). This evidence suggests that the dominant effect caused by the specific driver (such as EGFR or ALK in lung cancer(44)) makes tumors rely less on the accumulation of mutations. In the present study, we found that HCC carried frequent mutations in ATM and ATR, while BRCA1/2 was more predominant in ICC (Figure 2). The undefined driving or accompanying role of DDR mutations in different pathological types of PLC may also account for the different role of TMB in survival prognosis. More importantly, TMB may be the outcomes, not driving factors, from the oncogenic alterations which lead the poorer survival for some patients(45).
The leading dilemma of the DDR mutational situation in PLC is how to translate actionable alterations in DDR genes to achieve precision oncology. Considering the feasibility of using a PARPi compound to treat DDR mutated cancers, whether DDR$^{mut}$ PLC patients (particularly patients with ATM or BRCA1/2 mutations) are the optimal candidates for receiving PARPi should be explored. The present study revealed the therapeutic efficacy of olaparib as a post-second-line treatment in 7 advanced ICC patients and 1 H-ChC patient, suggesting the patients with BRCA1/2$^{mut}$ PLC (especially with germline mutations) should actively be considered for PARPi treatment. Our study's outcomes broaden the precision oncology for hepatobiliary tumors. We also noticed that the potential to benefit several (34.8%, 32/92) DDR$^{mut}$ PLC patients with actionable alterations seems to offer alternative targeted therapy except for that with PARPi. Co-occurring mutations in FGF14/IRS2/TNFSF13B/STK24 were observed in DDR$^{mut}$ PLC, these four genes mainly located at mitogen activated protein kinase (MAPK) pathway(46), which regulates many biological and physiological processes such as cellular proliferation, angiogenesis and cellular matrix formation. Importantly, MAPK pathway has firmly dynamic cross-talk with PI3K/AKT/mTOR pathway(47), and these pathways modulate cellular metabolism including glycolysis, lipid biogenesis and protein synthesis. Thus, co-inhibition targeting MAPK/mTOR pathway may be a strategy for treating liver cancer(48). However, it simultaneously brought confusion in how to set an appropriate standard or evidence level to determine the best treatment when two or more actionable alterations appeared; whether combinational treatment targeting multiple actionable targets is more effective; and how to combine targeted treatment with immunotherapy to achieve a synergistic effect. Another point of confusion is the discrepant response in identical treatment using olaparib, raising a major challenge to precision oncology as this field develops. Various factors might underlie the disparate efficacy in therapy: different mutational features in BRCA1/2 (Figure 3); somatic or germline mutations (Figure 3); differences in mutual or exclusive mutations; and discrepancies in chromosome and genetic instability.

In conclusion, in the present study, we identified the mutational landscape of DDR genes in patients with PLC. The positive correlation between DDR mutations and TMB...
level was confirmed in patients with PLC. Precision oncology based on actionable alterations was investigated in DDR\textsuperscript{mut} PLC, highlighting the translational significance of clinical treatment using a PARPi or an ICI. Further research should focus on disclosing the relationship between genotypes and phenotypes for DDR mutations in PLC to explain the cancer-driving or cancer-accompanying effects of diverse DDR mutations.
Figure legends

**Figure 1. Patterns and distributions of DDR mutations in primary liver cancers.** (A) Frequency of mutational types for DDR genes. (B) The distribution and numbers of DDR somatic mutations in each pathological subtype and in each individual DDR gene. (C) Number of patients with DDR germline mutations. (D-F) Family diagrams for three independent patients who carried definite susceptible loci of BRCA1/2; the dark dots indicate members with cancer, "W" refers to wild type at a locus, "M" refers to mutant at a locus, "P" refers to patients with primary liver cancer (intrahepatic cholangiocarcinoma) enrolled in our study (Patient IDs: Patient014, Patient051, Patient004).

**Figure 2. Associations of DDR mutations with tumor mutation burden (TMB) in primary liver cancer** (all TMB values have been transformed by log2). (A) Comparison of TMB levels among three different pathological subtypes regardless of mutant or wild-type DDR genes. (B) TMB stratified by DDR mutation status. (C) Comparison of TMB among three different pathological subtypes with DDR mutations. (D) Association of DDR mutation and related contributing factors with higher TMB in the study population (*: factor significantly related to TMB level). (E) Comparison of TMB among patients stratified by DDR mutation status and BFM mutation status, the TMB level of BFM mutants was significantly higher than others (Note: 26 patients belonged to both BFM group and DDR-nonBFM group because of some DDR genes simultaneously existed in different categories for DDR genes).

**Figure 3. BRCA1/2 mutational patterns in the study population and for patients who received PARPi (olaparib) treatment.** (A) Annotations and locations of mutated loci of BRCA1/2 in our cohort. The red dots indicate mutations that occurred at the germline level while the blue dots indicate somatic mutations. Loci with olaparib efficacy are highlighted, and loci related to partial response (PR), stable disease (SD) and progressive disease
(PD) are marked by the red rectangles, green rectangles and gray rectangles, respectively. **(B)** The summary for patients treated by PARPi (olaparib), including information about clinical features, therapeutic outcomes and mutational targets. Note: assessments for therapeutic response were according to Response Evaluation Criteria in Solid Tumors, RECIST, version 1.1).

**Figure 4. The landscape of cancer-related mutations, translational targets and actionable alterations in DDR\textsuperscript{mut} primary liver cancers.** **(A)** Oncoprint of select gene alterations, pathways and mutational patterns for DDR\textsuperscript{mut} primary liver cancers, separated by three different pathological types. Panel A shows the distribution of 8 selected DDR genes, with 5 DDR genes with high mutated frequency (ATM, BRCA2, BRCA1, MLH1 and ATR) and 3 DDR genes with biological significances (POLE, RAD50 and MSH2). For other functional pathways, genes with high mutated frequency were enriched into three leading and different pathways including TP53/cell cycle, chromatin-modifying and RTK-PIK3. Some alterations with important biological significances, such as STK24, FAT3/4, were also presented. **(B)** The distribution of cooccurring or exclusively occurring mutations in select genes for DDR\textsuperscript{mut} primary liver cancers. **(C)** The left pie-plot indicates the frequency of patients with DDR\textsuperscript{mut} PLC (N=55) or DDR\textsuperscript{wt} PLC (N=127) who were identified with translational targets in our cohort. The right pie-plot shows the distribution of OncoKB levels for translational targets in patients with DDR\textsuperscript{mut} PLC or DDR\textsuperscript{wt} PLC. **(D)** The flow diagram in the left part shows the list of translational targets for each OncoKB recommendation level in DDR mutant, and the right part presents for DDR wild type PLC. The colors of the curving belts represent different signaling pathways, and the widths of the belts indicate different frequencies for each target at every level. **(E)** The panel shows the comparison of actionable alteration frequencies between DDR\textsuperscript{mut} and DDR\textsuperscript{wt} primary liver cancers.
References


47. Favier J, Amar L, Gimenez-Roqueplo AP. Paraganglioma and phaeochromocytoma: from genetics to personalized medicine. Nature reviews Endocrinology 2015;11(2):101-11 doi 10.1038/nrendo.2014.188.

Figure 3.

A

BRCA1

- Zinc finger, C3HC4 type (RING finger)
- RING-finger (Really Interesting New Gene) domain
- Serine-rich domain associated with BRCT

BRCA2

- BRCA2 repeat
- BRCA2, helical
- BRCA2, oligonucleotide/oligosaccharide-binding, domain 1

B

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Pathology</th>
<th>Age</th>
<th>Sex</th>
<th>Mut Level</th>
<th>HGVSp</th>
<th>Response</th>
<th>PFS (months)</th>
<th>Previous Chemotherapy</th>
<th>OncoKB</th>
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<tr>
<td>Patient 051</td>
<td>ICC</td>
<td>41</td>
<td>M</td>
<td>Germline</td>
<td>p. Glu58Ter</td>
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<td>6</td>
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<td>Level 2B</td>
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<td>Patient 014</td>
<td>ICC</td>
<td>62</td>
<td>F</td>
<td>Germline</td>
<td>p. Glu335GlyfsTer2</td>
<td>PR</td>
<td>5</td>
<td>Gemcitabine</td>
<td>Level 2B</td>
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<tr>
<td>Patient 004</td>
<td>ICC</td>
<td>65</td>
<td>F</td>
<td>Germline</td>
<td>p. Ser2670Ter</td>
<td>PR</td>
<td>13</td>
<td>Gemcitabine</td>
<td>Level 2B</td>
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<tr>
<td>Patient 057</td>
<td>ICC</td>
<td>62</td>
<td>F</td>
<td>Somatic</td>
<td>BRCA2-FRY rearrangement</td>
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<td>3</td>
<td>Gemcitabine + Oxaliplatin</td>
<td>Level 2B</td>
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<tr>
<td>Patient 056</td>
<td>ICC</td>
<td>51</td>
<td>F</td>
<td>Somatic</td>
<td>p. Leu1390PhefsTer13</td>
<td>SD</td>
<td>4</td>
<td>Gemcitabine + Oxaliplatin</td>
<td>Level 2B</td>
</tr>
<tr>
<td>Patient 083</td>
<td>ICC</td>
<td>61</td>
<td>M</td>
<td>Somatic</td>
<td>p. Glu2571Asp p. Leu2581Ter</td>
<td>PD</td>
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<td>Capecitabine + Oxaliplatin</td>
<td>Level 2B</td>
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<tr>
<td>Patient 006</td>
<td>ICC</td>
<td>62</td>
<td>M</td>
<td>Somatic</td>
<td>p. Val2109Ile</td>
<td>PD</td>
<td>2</td>
<td>Gemcitabine; Afatinib</td>
<td>Level 2B</td>
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<tr>
<td>Patient 032</td>
<td>H-ChC</td>
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<td>M</td>
<td>Somatic</td>
<td>p. Arg979His (BRCA1)</td>
<td>PD</td>
<td>2</td>
<td>Capecitabine</td>
<td>Level 2B</td>
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</tbody>
</table>
### Tables

#### Table 1. Clinicopathological characteristics of the study population (N=357).

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<th></th>
<th>HCC (N=214)</th>
<th>ICC (N=122)</th>
<th>H-ChC (N=21)</th>
<th>ALL (N=357)</th>
<th>P-value</th>
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<tr>
<td><strong>Age (mean, range)</strong></td>
<td>54 (16-79)</td>
<td>59.5 (28-88)</td>
<td>58 (37-73)</td>
<td>56 (16-88)</td>
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</tr>
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<td><strong>Sex (Male)</strong></td>
<td>190 (88.8%)</td>
<td>72 (59.0%)</td>
<td>19 (90.5%)</td>
<td>281 (78.7%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Clinical Stage (≥III)</strong></td>
<td>71 (33.2%)</td>
<td>63 (51.6%)</td>
<td>8 (38.1%)</td>
<td>142 (39.8%)</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>Differentiation (moderate)</strong></td>
<td>78 (36.4%)</td>
<td>37 (30.3%)</td>
<td>4 (19%)</td>
<td>119 (33.3%)</td>
<td>0.3875</td>
</tr>
<tr>
<td><strong>HBV±HCV Infection</strong></td>
<td>168 (78.5%)</td>
<td>41 (33.6%)</td>
<td>14 (66.7%)</td>
<td>223 (62.5%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Infestation of Liver Fluke</strong></td>
<td>3 (1.4%)</td>
<td>2 (1.6%)</td>
<td>0</td>
<td>5 (1.4%)</td>
<td>0.998</td>
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<tr>
<td><strong>Liver Cirrhosis</strong></td>
<td>196 (91.6%)</td>
<td>48 (39.3%)</td>
<td>12 (57.1%)</td>
<td>256 (71.7%)</td>
<td>&lt;0.001</td>
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<tr>
<td><strong>Tissue Origins</strong></td>
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<td></td>
<td></td>
<td></td>
<td>0.15</td>
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<tr>
<td><strong>Primary</strong></td>
<td>199 (93.0%)</td>
<td>106 (86.9%)</td>
<td>19 (90.5%)</td>
<td>324 (90.8%)</td>
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<td><strong>Metastasis</strong></td>
<td>15 (7.0%)</td>
<td>16 (13.1%)</td>
<td>2 (9.5%)</td>
<td>33 (9.2%)</td>
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<tr>
<td><strong>Chemotherapy-naive</strong></td>
<td>188 (87.9%)</td>
<td>92 (75.4%)</td>
<td>17 (81.0%)</td>
<td>297 (83.2%)</td>
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<tr>
<td><strong>Family Cancer History</strong></td>
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<td>0.4759</td>
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<tr>
<td><strong>Yes</strong></td>
<td>75 (35.0%)</td>
<td>35 (28.7%)</td>
<td>7 (33.3%)</td>
<td>117 (32.8%)</td>
<td></td>
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<tr>
<td><strong>No</strong></td>
<td>97 (45.3%)</td>
<td>54 (44.3%)</td>
<td>8 (38.1%)</td>
<td>159 (44.5%)</td>
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<tr>
<td><strong>Unknown</strong></td>
<td>42 (19.6%)</td>
<td>33 (27.0%)</td>
<td>6 (28.6%)</td>
<td>81 (22.7%)</td>
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<tr>
<td><strong>Biliary Stone Disease</strong></td>
<td>78 (36.4%)</td>
<td>59 (48.4%)</td>
<td>11 (52.4%)</td>
<td>148 (41.5%)</td>
<td>0.06</td>
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</table>

**Note:** P-values indicate the statistical significances of the differences existed in three subtypes.

#### Table 2. Mutations of DDR genes and functional categories for PLC patients.

<table>
<thead>
<tr>
<th>Variables</th>
<th>HCC (N=214)</th>
<th>ICC (N=122)</th>
<th>H-ChC (N=21)</th>
<th>P-value</th>
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</thead>
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<tr>
<td><strong>Somatic DDR</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mut (%)</td>
<td>42 (19.63)</td>
<td>29 (23.77)</td>
<td>6 (28.57)</td>
<td>0.456</td>
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<tr>
<td><strong>Germline DDR</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mut (%)</td>
<td>7 (3.27)</td>
<td>8 (6.56)</td>
<td>0</td>
<td>0.273</td>
</tr>
<tr>
<td><strong>Functional Categories</strong></td>
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<tr>
<td>BER</td>
<td>14 (6.54)</td>
<td>6 (2.80)</td>
<td>1 (4.76)</td>
<td>0.864</td>
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<td>FA</td>
<td>11 (5.14)</td>
<td>13 (10.66)</td>
<td>4 (19.05)</td>
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<td>MMR</td>
<td>10 (4.67)</td>
<td>7 (5.74)</td>
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<td>0.708</td>
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<td>HRR</td>
<td>8 (3.74)</td>
<td>18 (14.75)</td>
<td>1 (4.76)</td>
<td>0.001</td>
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<td>CPF</td>
<td>20 (9.35)</td>
<td>6 (4.92)</td>
<td>1 (4.76)</td>
<td>0.314</td>
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<tr>
<td>NHEJ</td>
<td>3 (1.40)</td>
<td>5 (4.10)</td>
<td>0</td>
<td>0.298</td>
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</table>

**Abbreviations:** BER, base excision repair; CPF, checkpoint factors; FA, Fanconi anemia; HRR, homologous recombination repair; MMR, mismatch repair; NHEJ, nonhomologous end joining.

**Note:** P-values indicate the statistical significances of the differences existed in three subtypes.
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