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Identification of driver genes and target drugs-related genes in liver cancer based on targeted next generation sequencing

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The purpose of this study was to identify gene mutations, high frequency mutations, and driver genes in liver cancer, and the marketed approved drugs of these genes, to provide evidence for targeted treatment of liver cancer since it is one of the most common cancers worldwide. 34 patients with liver cancer were included, and their blood samples were collected. The pathway enrichment analysis of the mutation gene was carried out through the KEGG database, and the genes with marketed approved drugs were screened according to the Pharmalaxy database. A total of 6,612 mutations in 1,241 genes were identified in 34 patients, in which 22 genes mutated in at least 40% of the samples and were thought to be high frequency mutation genes. All the mutations were analyzed using the MutSigCV software, and 30 genes with $q<0.1$ and $p<0.05$ were selected out as driver genes. Among them, LRP1B, MYC, NF1, and KEAP1 were coincident with high frequency mutation genes, which were considered key driver genes. Afterward, 181 genes with $p<0.05$ in MutSigCV software were analyzed for pathway enrichment. These genes were mainly enriched in four pathways, including MAPK mTOR, p53/cell cycle, and JAK-STAT pathways. Finally, there were 15 genes in four pathways that had marketed approved target drugs. To conclude, LRP1B, MYC, NF1, and KEAP1 were the candidate key driver genes for liver cancer, which might provide new insights for targeted therapy of liver cancer.

Key words: liver cancer, gene mutation, driver gene, targeted therapy

China has the highest incidence of liver cancer in the world, accounting for about 50% of new cases and related deaths of liver cancer in the world [1]. Different from other malignant tumors with multiple genetic variation markers that determine tumor behavior, liver cancer is a solid tumor with complex molecular pathogenesis, and its occurrence and development involve the disorder of multiple pathways [2, 3]. The gene variation spectrum of hepatoma cells is wide and lacks clear genetic variation characteristics [4]. The large number and wide range of mutation genes and epigenetic variation genes increase the complexity of accurate molecular pathological diagnosis of liver cancer and enhance the difficulty of targeted treatment research, which is the biggest challenge for accurate medical research of liver cancer.

It is a very important task to study the function of a driving gene in a tumor. Thousands of sonic mutations coexist in tumors and only some of them will promote the development of tumors. This part of mutation is usually called driver mutation and the rest is called passenger mutation [5]. In recent years, with the rapid development of sequencing technology, genomics of liver cancer has made great progress, which provides new technical means for screening gene markers of clinical diagnosis and treatment, and prognosis [6]. The purpose of this study was to detect the mutation genes in blood samples and tissue samples of patients with liver cancer by high-throughput sequencing, identify the driver mutations and screen the target drugs of these mutated genes, so as to provide the basis for revealing the molecular mechanism and targeted treatment of liver cancer.

Patients and methods

Sample collection. 34 patients with liver cancer were enrolled, of which 26 were males and 8 were females, 7 were at stage I/II and 27 were at stage III/IV, age from 31 to 80 (average 57.97) years old. Their whole blood samples were

collected and sequenced using targeted next generation sequencing (NGS), while paracancerous tissues or leukocytes were used as controls. Informed consent had been obtained from all participants. This research was approved by the Ethical Committee of Tianjin Medical University Cancer Institute and Hospital.

Targeted NGS gene panel sequencing. A genomic DNA extraction kit (Qiagen, Hilden, Germany) was used to extract DNA from fresh tissue, and circulating free DNA (cfDNA) in plasma samples was extracted using the QIAamp Circulating Nucleic Acid Kit (Qiagen) according to the manufacturer's instructions.

Library preparation for each sample was performed following the manufacturer's protocol. Briefly, ~1 μg extracted DNA from fresh tissue was randomly sheared into 150–200 base pair fragments using the Covaris M220 instrument (Woburn, MA, USA), followed by library construction using KAPA Hyper DNA Library Prep Kit (KAPA Biosystems, Wilmington, MA, USA). The amount of cfDNA extracted was low (100–200 ng) without interruption (shear). Size selection was performed using Agencourt AMPure XP beads (Agencourt Biosciences, Beverly, MA, USA), followed by PCR amplification.

Targeted enrichment was performed using SeqCap EZ Prime Choice Probes (Roche, Basel, Switzerland), which captured a total of 1.1 Mb from 1,000 known cancer-related genes. The obtained libraries were subjected to targeted sequencing using an Illumina HiSeq Xten sequencer (San Diego, CA, USA).

Mutation analysis. The sequencing data were mapped to the hg19 reference genome with Burrows-Wheeler Aligner software for tumor-specific somatic mutation detection. Afterward, VarScan version 2.4.3, MuTect version 1.1.4, and GATK version 2.3.9 were used to detect variations of single nucleotide variants (SNV) and insertion-deletions (InDel). The selection criteria are depth >300, mutation frequency >0.5%.

Driver gene identification. According to all variation genes, the driver genes of HCC were analyzed using MutSigCV version 1.41 software. The cut-off criterion was $p<0.05$.

Functional annotation. For the mutated genes, pathway enrichment analysis and gene visualization were performed using Database for Annotation, Visualization and Integrated Discovery version 6.7 (http://david.abcc.ncifcrf.gov/) and KEGG PATHWAY (http://www.genome.jp/kegg) databases. A p-value <0.05 was the cut-off criterion.

Targeting drug-related genes. Through the Pharmacodia database (https://data.pharmacodia.com), the genes with approved targeting drugs and the corresponding genes were screened from the identified mutation genes.

Results

Mutation gene analysis. In 34 patients, a total of 6,612 mutations in 1,241 genes were found, including several mutation types such as indel, frameshift, missense, stop loss, nonsense mutation, etc. Among them, 22 genes were mutated in at least 40% of the samples and could be seen as high frequency mutation genes. Figure 1 showed the mutation distribution of 22 genes in all samples. Among them, silent mutation accounted for 21.54% and non-silent mutation accounted for 78.46%. In non-silent mutations, missense mutation was the most frequent (87.90%), next was frameshift (4.40%), followed by splice (3.72%), nonsense (3.08%), CDS-indel (0.59%), and other nonsynonymous mutations (0.31%). In the 22 genes, q values of LRP1B, MYC, NF1, and KEAP1 were less than 0.1, indicating a more significant mutation of the four genes.

Screening of driver genes. There were in all 181 genes with p-value <0.05 and the top 30 genes were selected according to the p-value and with the q value <0.1 (Table 1), these genes could be considered as driver genes. Figure 2A showed the

Table 1. The top 30 most significant mutated genes in 34 liver cancer patients.

Gene	p-value	q-value	Freq (%)	Gene	p-value	q-value	Freq (%)
SYK	5.85E–10	1.10E–05	35.29	EPHA5	2.38E–05	2.50E–02	32.35
BAP1	4.05E–09	2.54E–05	32.35	JAK1	2.19E–05	2.50E–02	23.53
KEAP1	2.75E–09	2.54E–05	41.18	MTOR	2.10E–05	2.50E–02	26.47
MYC	1.57E–08	7.40E–05	47.06	AXL	2.54E–05	2.52E–02	26.47
IDH2	5.46E–08	2.06E–04	32.35	AKT3	4.28E–05	4.04E–02	17.65
CDKN2B	7.99E–08	2.51E–04	20.59	MCL1	5.24E–05	4.52E–02	11.76
FGFR1	4.11E–07	1.11E–03	26.47	NF1	5.27E–05	4.52E–02	44.12
SMO	5.50E–07	1.30E–03	29.41	LRP1B	5.69E–05	4.67E–02	58.82
ZNF703	3.21E–06	6.72E–03	14.71	KDR	7.19E–05	5.65E–02	32.35
EPHA2	6.61E–06	1.17E–02	35.29	AKT2	8.28E–05	5.78E–02	17.65
FGFR2	6.82E–06	1.17E–02	23.53	DEK	8.16E–05	5.78E–02	14.71
FLT4	9.12E–06	1.43E–02	29.41	KIT	8.03E–05	5.78E–02	20.59
IGF1R	1.61E–05	2.34E–02	26.47	MAPK3	8.89E–05	5.99E–02	17.65
PTCH2	1.82E–05	2.45E–02	20.59	CDH1	1.17E–04	7.62E–02	20.59
CDKN1B	2.38E–05	2.50E–02	11.76	SMARCB1	1.31E–04	8.23E–02	17.65

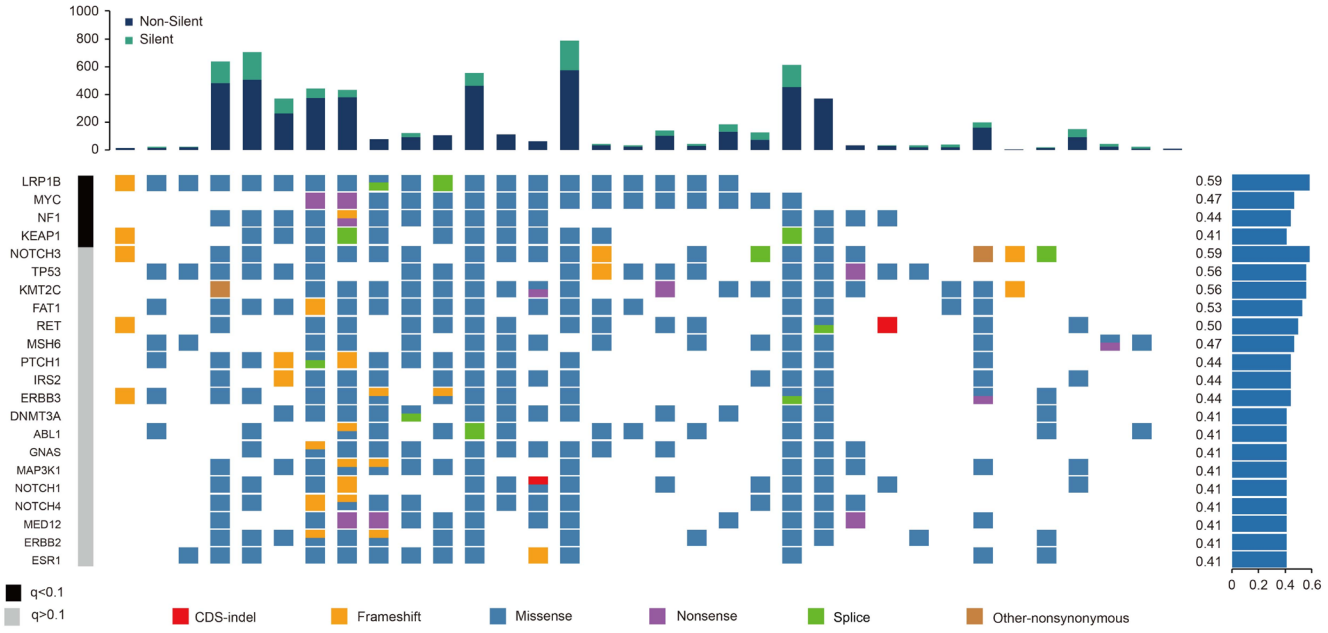

Figure 1. Mutation landscape of 22 genes mutated in more than 40% of liver cancer samples, with different colors representing different mutation types. The histogram on the right showed the frequency of mutations in 34 patients. The histogram above showed the distribution of silent and non-silent mutations in each sample.

alteration frequency, –log10 (MutSig p-value) and –log10 (MutSig q-value) of the 30 genes. Among them, SYK (9.23), BAP1 (8.39), KEAP1 (8.56), MYC (7.80), and IDH2 (7.26) were the top five genes of –log10 (MutSig p-value), as well as –log10 (MutSig q-value), indicating that the mutations of these genes have significant differences. Figure 2B showed the frequency of different mutation types of the 30 driver genes in 34 patients, the mutation frequency and type of different genes were quite different. In addition, the four genes of LRP1B, MYC, NF1, and KEAP1 were the first 4 genes with the highest mutation frequency in 34 patients (58.82%, 47.06%, 44.12%, and 41.18%, respectively), which could be considered as candidate key driver genes in lung cancer.

Pathway enrichment analysis. The 181 genes with $p<0.05$ were enriched in 55 pathways, including the mTOR signaling pathway, B cell receptor signaling pathway, MAPK signaling pathway, and p53 signaling pathway, etc. We summarized the main enrichment pathways, including MAPK, mTOR, p53/cell cycle, and JAK-STAT signaling pathways (Figure 3), which enriched 67.65%, 55.88%, 76.47%, and 52.94% of patients, respectively. MYC was a downstream gene in the MAPK pathway and a key node of this pathway, with a mutation frequency of 47.06%. MYC protein inhibited the expression of CDKN2A and CDKN2B proteins in the p53/cell cycle pathway, thereby inhibiting the expression of TP53. In addition, the expression of CDKN1A and CDKN1B in the p53/cell cycle pathway was inhibited by TP53 expression and AKT2 in the mTOR pathway, respectively.

Identification of target drug-related genes. Among the genes enriched in the above four pathways, there were 15 genes targeting marketed approved drugs, namely NF1, FGFR1, FGFR2, EGFR, PRKCG, MAP2K1, PRKCB enriched in the MAPK pathway; STAT3, JAK1, KIT in JAK-STAT pathway; ERBB4, MTOR, PIK3CA in the mTOR pathway; and TP53 and CDK6 in the p53/cell cycle pathway. Figure 4 showed the alteration types and frequencies of the 15 genes and their enriched pathways and targeted drugs, in which Sorafenib is the only approved target drug in liver cancer and its targeted gene is KIT. Other genes may be new strategies for the targeted treatment of liver cancer.

Discussion

In this study, we first identified the mutations in 34 liver cancer samples, 181 of which were $p<0.05$. In at least 40% of the samples, there were 22 mutated genes, such as TAP1, ANKRD11, NSD1, and CDK13, which were considered as high-frequency mutated genes. Afterward, 30 genes with significant mutations were selected based on q value <0.1. The role of 25 genes in liver cancer has been reported in previous studies: SYK, BAP1, KEAP1, MYC, IDH2, CDKN2B, FGFR1, SMO, EPHA2, FGFR2, FLT4, IGF1R, CDKN1B, EPHA5, JAK1, MTOR, AXL, AKT3, MCL1, NF1, KDR, AKT2, DEK, CDH1, and SMARCB1. The other five genes have not been reported in liver cancer: ZNF703, PTCH2, LRP1B, KIT, and MAPK3. Among them, LRP1B, MYC, NF1, and KEAP1 were

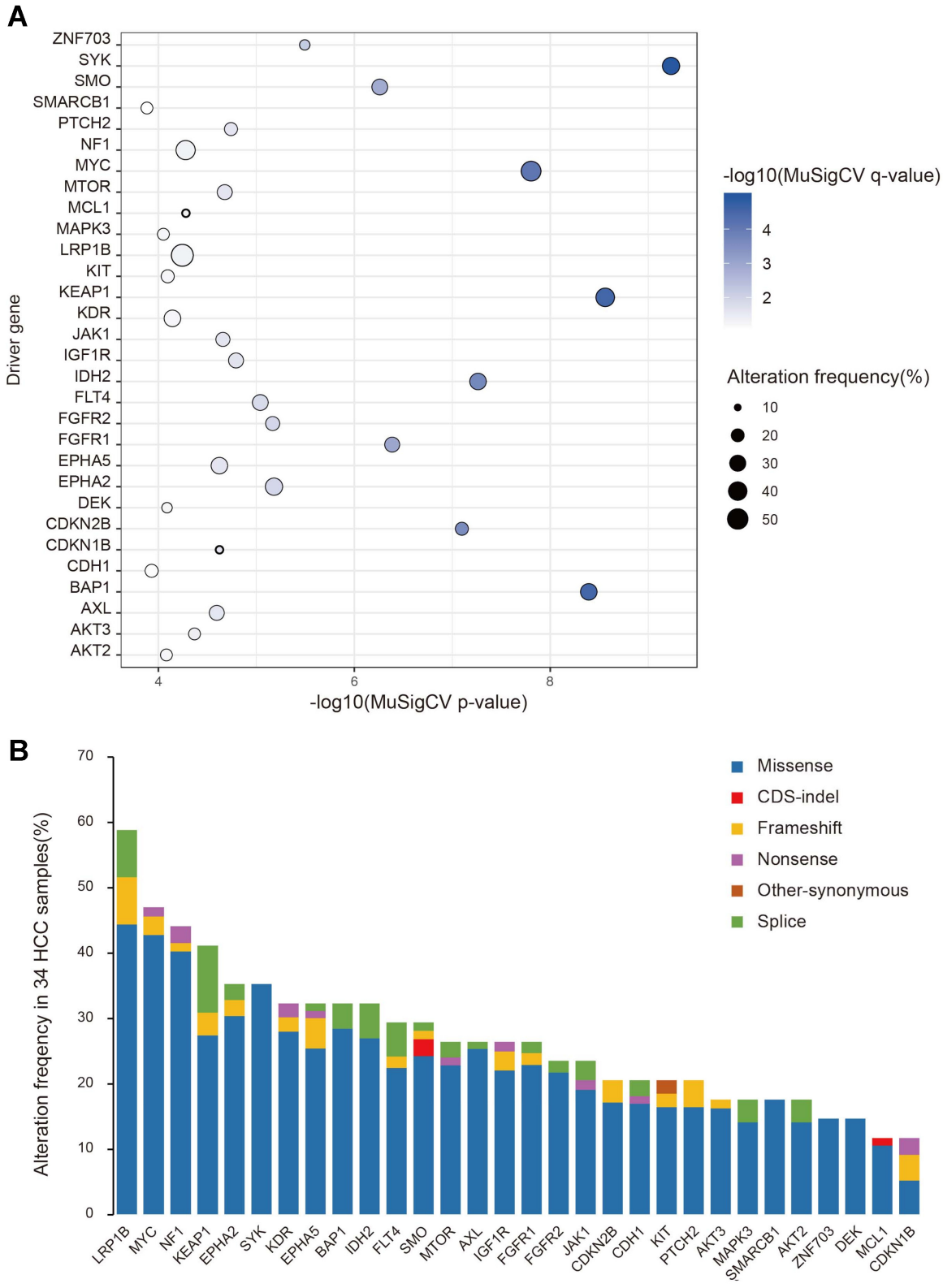

Figure 2. Identification of significantly mutated genes. A) 30 mutant genes with q<0.1, and has a log-transformed mutation significance on the x-axis. The size and color of each sample represent the frequency of change and the –log10 (MutSig q value), respectively. B) The histogram refers to the mutation frequency and type of the top 30 significant mutant genes.

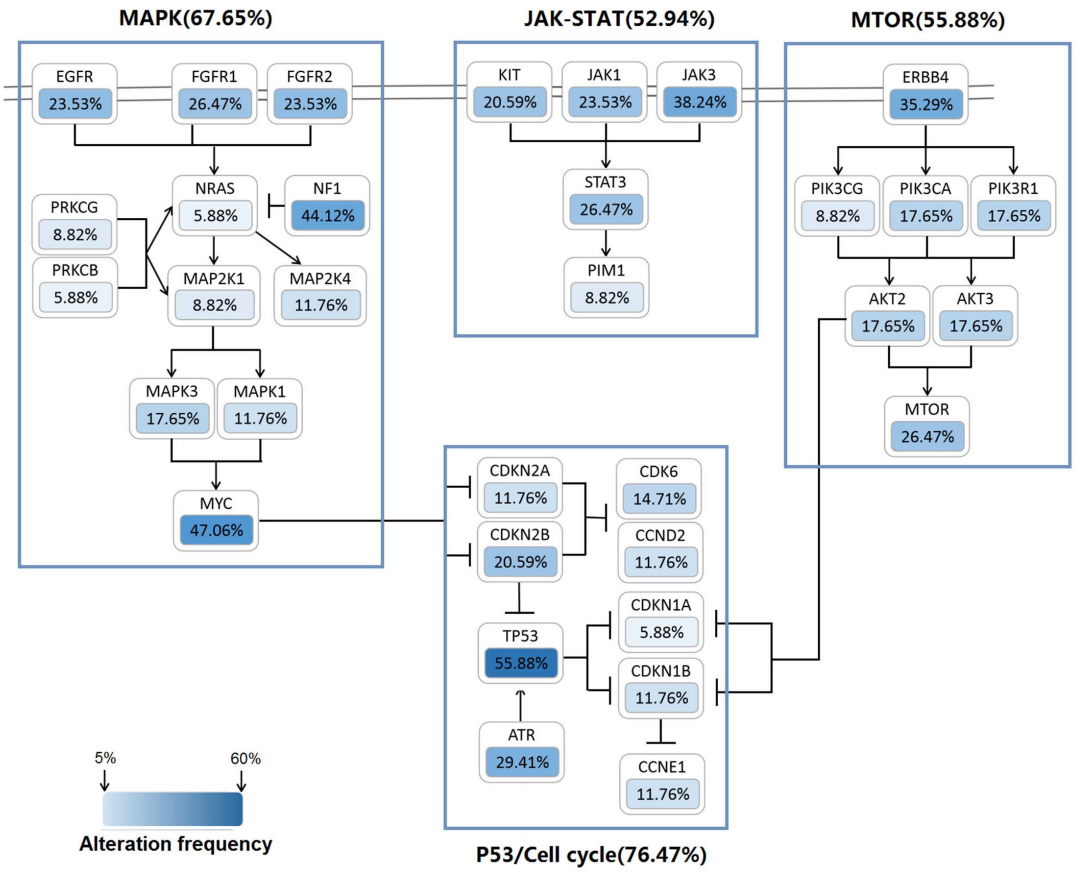

Figure 3. The enriched pathway terms of 181 significantly mutated genes.

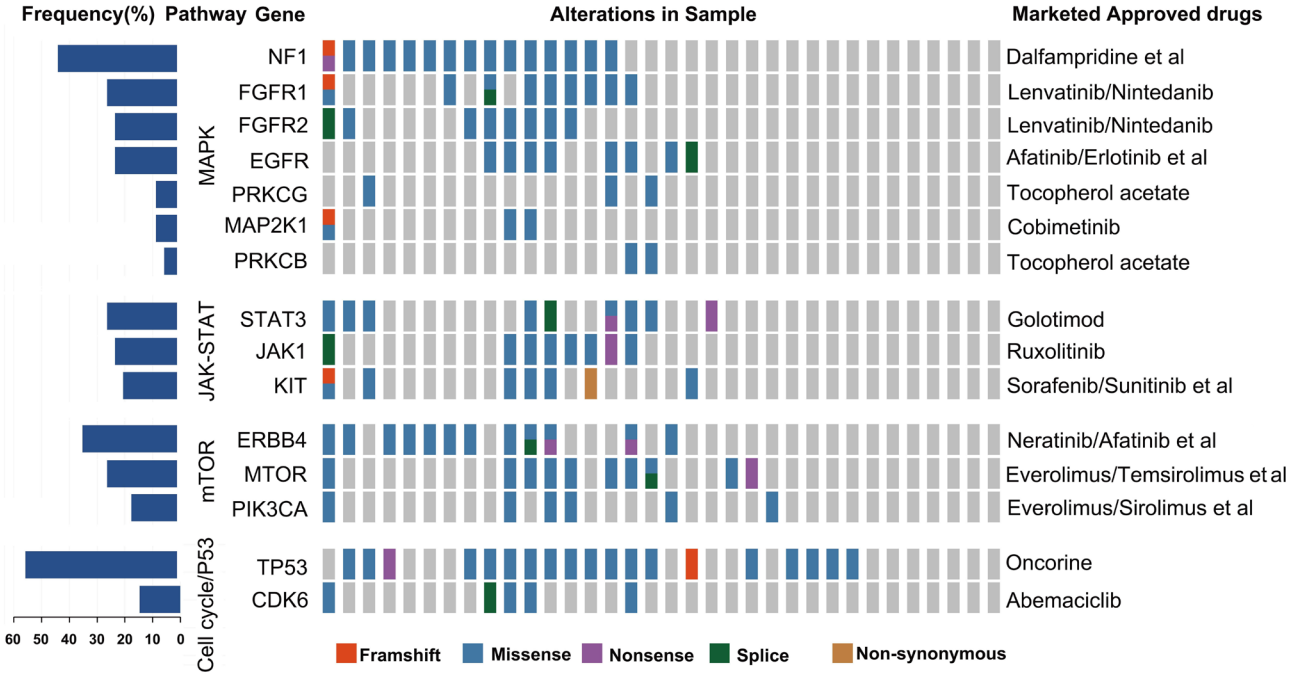

Figure 4. Mutation frequency and enriched pathway drugs in mutated genes targeted to marketed approved drugs, different colors represent different mutation types. The histogram on the left showed the mutation frequency of the gene, and on the right side are gene-targeted drugs.

all contained in high-frequency mutation genes and significant mutation genes, which were considered as candidate key driver genes of liver cancer.

In this study, LRP1B was the most frequently mutated gene (58.82%). LRP1B is a member of the low-density lipoprotein receptor family, which is involved in different functions of the human body, including cholesterol metabolism and the formation of atherosclerotic lesions. LRP1B was originally discovered when studying lung cancer cell lines [7]. In nearly 50% of non-small cell lung cancer (NSCLC) cell lines, changes to the LRP1B gene are part of the homozygous exon deletions or transcript deletions. It has been found that LRP1B is inactivated in many malignant tumors, such as hepatobiliary tumors, esophageal cancer, colon cancer, and gastric cancer, etc [8–11]. Therefore, LRP1B may be a potential tumor suppressor gene whose dysregulation is associated with cell migration, drug resistance, and poor clinical outcomes in cancer [12, 13]. LRP1B is also considered a potential driver gene, and its mutations are significantly associated with smoking in lung adenocarcinoma [14]. Wang et al. [15] found that the mutation rate of the LRP1B gene in liver cancer was 9.3% (35/377) and 7.8% (51/655) in the TCGA cohort and clinical cohort respectively, LRP1B mutations were significantly associated with high TMB. In addition, patients with LRP1B mutations in the TCGA cohort had significantly reduced overall survival (OS) and progression-free survival (PFS). The results show that LRP1B mutation is a poor prognosis factor for liver cancer, patients with liver cancer carrying this gene mutation may be more likely to benefit from immunotherapy. Subsequent confirmatory research deserves further development. MYC genome amplification and/or overexpression is a common molecular event in HCC [16]. MYC is a transcription factor that regulates thousands of genes that regulate their cancer signatures [17]. Studies have shown that inactivation of the MYC oncogene in HCC was sufficient to cause tumor regression associated with the arrest of proliferation, differentiation, and apoptosis. Therefore, MYC-based therapies may become an effective treatment for liver cancer. ctDNA sequencing of 14 patients with advanced HCC showed that MYC (n=2) mutation accounts for 14% of all patients [18]. Currently, many researchers are trying to find drugs that target MYC to treat cancer, but Lai et al. [19] proposed that cancers caused by MYC may be sensitive to immunotherapy because the emergence of MYC plays an important role in suppressing anti-tumor immune responses. The NF1 gene is a tumor suppressor gene located at 17q11.2. NF1 gene encodes neurofibrin, which is a functional Ras GTPase activating protein (RasGAP), can negatively regulate Ras signals by accelerating the conversion of activated Ras-GTP to inactive Ras-GDP. NF1 gene germline mutation can cause neurofibromatosis type 1 [20]. To date, more than 2,900 pathogenic variants of the NF1 gene have been reported in the human gene mutation database, but there are no obvious mutation hot spots. Approximately 50% of NF1 cases are sporadic and presented as new mutations [17]. NF1 mutations have been reported in 10% of HCC samples, such tumors can be sensitive to the PI3K/AKT/mTOR and MAPK inhibitors [21]. Keap1 gene is located on human chromosome 19p13.2 and encodes 624 amino acids. Keap1 is currently thought to be a binding protein of nuclear factor erythroid 2-related factor 2 (Nrf2) in the cytoplasm and is anchored in the cytoplasm with actin-binding [22]. Keap1 participates in the protective mechanism of cells against endogenous and exogenous active oxygen and electrophilic substances to damage cells through the Keapl-Nrfs2 signaling pathway and plays an important role in antioxidant, anti-stress, anti-tumor, anti-inflammatory [23]. Keap1 acts as a chaperone molecule in the cytoplasm to bind to the transcription factor Nrf2 and promotes ubiquitination and degradation of Nrf2. Keap1-Nrf2 is an important signaling pathway existing in cells. This pathway can participate in the regulation of inflammation and the regulation of the expression of antioxidant genes. Extensive cytoprotective functions have significant effects on chemoradiotherapy [24]. The differential expression rate of KEAP1 in lung adenocarcinoma and lung squamous cell carcinoma was statistically significant ($p<0.05$) [25]; in hepatocellular carcinoma, NFE2L2 and its interaction factor KEAP1, which are important in cellular antioxidant defense, were at 3% and a significant mutation occurred in 5% of HCC [26]. Yoo et al. detected somatic mutations of KEAP1 in gastric cancer, hepatocellular carcinoma, colorectal cancer, lung cancer, breast cancer, and the double allele inactivation in KEAP1 and increased levels of cytoprotective proteins in cancer suggest that KEAP1 mutations may protect cancer cells from oxidative damage and play a role in the development of solid cancers [27].

To identify cellular pathways associated with liver cancer, we used the KEGG database to enrich the pathways for 181 mutant genes with $p<0.05$. These genes are mainly concentrated in four pathways (Figure 3), including MAPK pathway, mTOR pathway, p53/cell cycle pathway, and JAK-STAT pathway. Mitogen-activated protein kinase (MAPK) has three members in mammals: ERK, p38, and JNK. JNK has been reported to activate NF-κB signaling by directly phosphorylating IκB kinase. In addition, MAPK is involved in activating activator protein 1, activating transcription factors 2, cyclic adenosine response element-binding protein, and other key signaling molecules activated by a variety of transcription factors [28]. In HCC, the MAPK signaling pathway is also a downstream signaling pathway for targets such as VEGFR and PDGFR [29]. mTOR consists of two compounds with different functions and structures, called mTORC1 and mTORC2, respectively. mTORC1 can directly phosphorylate the downstream targets of S6K1 and 4E-BP1, thus enhancing the translation of multiple mRNAs. The mTOR pathway and abnormal activation occur in up to 50–60% of HCC cases, leading to liver cancer, and are related to less differentiated tumors, early recurrence, and poor prognosis. Therefore,

blocking the mTOR pathway may be an effective treatment strategy for HCC. A recent study showed that in some cases, AKT activation through mTORC1 inhibition and mTORC2 contribute to drug resistance. Therefore, it can be imagined that targeting mTORC1 and mTORC2 in cancer will provide more effective antitumor activity [30]. p53 is known to be highly related to the apoptotic pathway and contributes to the anti-cancer process. CD147 promotes the proliferation of HCC cells by inhibiting the p53-dependent signaling pathway [31]. miR-221 maintains cell cycle progression and apoptosis response to adriamycin in HCC derived cell lines by regulating the p53/MDM2 feedback loop. Therefore, p53 is an important regulator in tumor progression [32]. The JAK-STAT signaling pathway is involved in cell proliferation, apoptosis, migration, differentiation, immune regulation, and other physiological processes, and is activated in a variety of tumor cells [33]. Studies have shown that the JAK-STAT signaling pathway plays an important role in the proliferation and apoptosis of breast cancer cells, and the inhibition of this signaling pathway can inhibit the occurrence and development of cancer cells [34]. In our study, the genes in each of the four pathways were mutated in more than 50% of the population, indicating that they may play a potential role in the occurrence and development of liver cancer.

Finally, we screened the genes that are enriched in the above four pathways and have marketed approved target drugs, and 15 genes were obtained. Among the four genes of LRP1B, MYC, NF1, and KEAP1, NF1 gene had marketed approved drugs, including dalfampridine et al. Other target drugs were Lenvatinib, Nintedanib, Afatinib, Erlotinib et al. (Figure 4). For all the identified drugs, Sorafenib is the only drug approved by the Food and Drug Administration (FDA) for the first-line treatment of advanced primary liver cancer [35]. At present, LRP1B, MYC, and KEAP1 have no approved targeted drugs in liver cancer, they may be potential targets and provide new ideas and directions for the research of targeted therapy of liver cancer.

This study identified more high-frequency mutations and driver genes in patients with liver cancer, as well as pathways enriched for these mutations and their target drugs that have been marketed. After screening, LRP1B, MYC, NF1, and KEAP1 may be the key driver genes for liver cancer. Among them, the target drug of NF1 is dalfampridine, and there are no marketed approved target drugs for the other three genes, which could be potential targets for liver cancer treatment. It provides a comprehensive understanding and new insights into future research on targeted therapy of liver cancer.

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