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박 사 학 위 논 문

Characteristics of Recurred Hepatocellular Carcinoma

- Based on Serum Biomarkers and Genetic Mutations -

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이 논 문 을 박 사 학 위 논 문 으 로 제 출 함

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2025년 2월

조 인 수

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1. Introduction

In the diagnosis of hepatocellular carcinoma (HCC), imaging studies such as computed tomography (CT) and magnetic resonance imaging (MRI) are essential for confirmation, whereas serum tumor markers are primarily utilized for disease screening and prognostic assessment. Although assessments using a single marker exhibit limited sensitivity in detecting both early-stage and advanced HCC, the combined use of these markers may enhance the sensitivity and specificity for diagnosing and prognosticating HCC.(1, 2) Owing to the heterogeneous nature of HCC, the number of elevated tumor markers varies among tumors. Approximately 20% of patients exhibit elevated levels of both AFP and PIVKA-II, while 40% show an elevation in only one of these markers, and another 20% maintain normal levels of both markers.(3, 4) Some studies indicate that variations among patients may reflect differences in clinicopathologic characteristics based on tumor marker profiles.(5-7)

Recent advances in genomic analysis have led to extensive research aimed at identifying key genetic alterations associated with the development and progression of HCC. Mutations in the TERT promoter, TP53, and CTNNB1, along with activation of pathways associated with EGFR, TGFB, VEGF, and tyrosine kinase receptors, are identified as significant contributors to HCC pathogenesis.(8, 9) Variations in these mutations among patients define the molecular subtype of the tumor and are associated with different prognoses.(8, 10, 11)

Genetic features, such as driver gene mutations, reflect the tumor characteristics of hepatocellular carcinoma (HCC). We have shown that different serum tumor marker levels correspond with distinct genetic

profiles, suggesting that tumor genetic characteristics could potentially be predicted using simple blood tests.(4) HCC recurrence after primary treatment is common, and various treatment modalities can be applied akin to primary cancer. Intrahepatic metastasis (IM), which shares similar biology with primary cancer (12, 13), is associated with poor prognosis. In contrast, multicentric occurrence (MO, de novo mass), which exhibits different biology from primary cancer, may have a better prognosis. Understanding the molecular biology of recurrent HCC could aid in selecting effective treatment strategies. However, due to cost constraints, gene sequencing cannot be performed for all patients. Currently, no clinical method exists to determine whether a recurrent tumor is IM or MO at the time of recurrence. (14, 15)

We hypothesized that serum tumor marker patterns of primary and recurrent masses could reflect the genetic characteristics of both primary and recurrent tumors. To test this hypothesis, we conducted a study to examine the correlation between serum biomarker patterns and the genetic profiles of primary and recurrent HCC.

2. Material and Method

2.1 Study population.

This study encompassed 20 patients from our institution who underwent a second surgery for recurrent hepatocellular carcinoma (HCC) following their initial surgery between 2011 and 2021, with frozen tumor tissues from both the primary and recurrent cancers available for analysis. Serum AFP and PIVKA-II levels were measured before the initial and second surgeries in all patients. DNA mutations were analyzed using next-generation sequencing on the obtained tumor and adjacent normal liver tissues. The institutional review board at Keimyung University Dongsan Hospital approved this study [2020-03-013-013].

2.2 Group discrimination for analysis

Patients were categorized into 4 groups based on the number of elevated tumor markers measured before the first and second surgeries. Group A was defined by patients with elevated AFP and normal PIVKA-II levels. Group B involved patients with elevated PIVKA-II levels and normal AFP. Group C included patients with both tumor markers elevated, and Group D consisted of patients with normal levels of both markers. (Figure 1) Patients' tumor markers were divided at cutoffs of 20ng/mL for AFP and 40ng/mL for PIVKA-II according to our previous study.(4) By comparing tumor marker groups from the first and second surgeries, patients in the same group were classified into

the serum concordant group, while those in different groups were placed into the serum discordant group. Upon comparing the genetic profiles of primary and recurrent tumors, patients were categorized into the genetic concordant group if they had the same genetic mutation in both surgeries. If patients had different genetic mutations in the two surgeries, they were included in the genetic discordant group.

2.3 Adaptor preparation for suppression PCR

Targeted sequencing with SMiLE-seq (Standwise and Molecularly-indexed Landmark Enrichment sequencing) was employed to analyze gene alterations. A 71-nucleotide-long oligonucleotide (5'-AGGACCGTGTGCTGACACTCTTTCCCTACACGACGCTCTTCCGA TCTNNNNNNNNCAGCTGACGTCAGTCT-3') containing a molecular index of eight random nucleotides was hybridized to a shorter, 5'-phosphorylated oligonucleotide (Pi-GACTGACGGCAGCTG-3') to create the SupL5 adaptor, which features a partial duplex region with a single mismatched base pair next to the molecular index. Hybridization was conducted by heating the oligonucleotide pair to 95 °C for 5 minutes, followed by stepwise cooling to room temperature at a rate of 1 °C per minute. Subsequently, the partial duplex DNA underwent a polymerization reaction to fill the single-stranded region, including the molecular index, using Klenow Fragment (3'→5' exo-) (NEB, USA) at 37 °C for 20 minutes. The SupL5 adaptor was then prepared by purifying the fully duplexed DNA, except for the 3' T overhang and mismatch, using ExpinTM PCR SV (GeneAll, Korea), and dissolving it in elution buffer to achieve a concentration of 16 µM.

2.4 Primer preparation

Primers were designed to optimize the enrichment of the coding regions of the target genes. A list of primer candidates was generated using Primer3 (Untergasser A et al., 2012) within the 400 bp regions adjacent to the BstNI site or within regions flanked by BstNI sites, encompassing the coding sequences of the target genes. Two primers with identical orientation were chosen for each target coding region for nested suppression PCR based on the following criteria: 1) primer binding sites in the reference genome should not exceed three when allowing for a single-base mismatch, 2) primers covering larger coding regions were prioritized, and 3) the calculated melting temperature should be no more than 3°C away from the average. The primers were organized into four groups according to their use (first or nested PCR) and their orientation with respect to the reference genome sequence: first forward, first reverse, nested forward, and nested reverse. In total, 2724 primer pairs were synthesized for 167 cancer-related genes.

2.5 Library Preparation for Precision Targeted Sequencing

One hundred nanograms of genomic DNA was digested with 10 units of BstNI restriction enzyme (NEB) at 60 °C for 4 hours. The fragmented DNA was then purified using Expin™ PCR SV (GeneAll, Korea) according to the manufacturer's instructions and eluted in 30 µL of elution buffer. The purified DNA underwent end-repair and A-tailing in

a reaction containing dNTP and SolgTM Taq polymerase (Solgent, Korea) at 65 °C for 30 minutes, which was then purified using DNA Clean & ConcentratorTM-5 (Zymo Research) following the manufacturer's instructions and eluted in 6 μ L of elution buffer. The entire volume of the 3'A-tailed DNA was mixed with 1 μ L of 16 μ M SupL5 adaptor and 7 μ L Blunt/TA ligation Master Mix (NEB), and incubated at room temperature for 20 minutes. This ligation reaction was subsequently purified again using ExpinTM PCR SV and eluted in 40 μ L of elution buffer. 2 μ L of the adaptor-ligated DNA served as the template for the nested PCR reaction, which utilized two sets of primer pairs: one specific for the target sequences and the other for the adaptor sequence. The first PCR reaction was conducted using SolgTM h-Taq polymerase, initiating with polymerase activation at 95°C for 15 minutes followed by 20 cycles of 95°C for 10 seconds, 58°C for 30 seconds, and 68°C for 1 minute. The second nested PCR used 1 μ L of the product from the first PCR, with polymerase activation at 95°C for 15 minutes followed by 10 cycles of 95°C for 10 seconds, 58°C for 30 seconds, and 68°C for 1 minute. Finally, an aliquot of the Nested PCR product underwent an additional 10 cycles of PCR using sample index primers to add a sample index and to form the sequencing library structure for the Illumina platform.

2.6 Sequence analysis

Sequences of paired-end reads, structured with a molecular index, short duplex sequence with strand mark, and one target sequence in the 5' to 3' order, were selected. The details regarding the library name, read

identifier, and the combination of molecular index and strand mark were stored separately. Among the unique sequences collected, those appearing more than twice across all libraries were aligned to the human reference genome, CRCh38/hg38, using the following BLAST command and options: `blastn-query QUERYFILE -db REFERENCE -perc_identity 95 -max_target_seqs 5 -max_hsps 5 -outfmt "6 qseqid sseqid pident length mismatch qstart qend sstart send sstrand btop eval evalue bitscore" -out OUTFILE`. The resulting BLAST output was analyzed, and sequences were mapped to the locus with the highest bitscore. If multiple loci shared the same bitscore, a representative locus was selected based on the earliest chromosome number and position. Using these mapping results, reads from each library were organized by their molecular index and original strand at reference loci to construct a single-strand consensus, followed by a double-strand consensus for those indices. Effective double-strand consensus sequences were those built from two single-strand sequences, each supported by at least two reads. For each molecular index, double-strand consensus sequences were compiled for each mapped locus from heptaplicated libraries, and a list of variant sequences was created. Positions with discrepancies between the two strands were deemed ineffective. Variants located within a single nucleotide homopolymer of more than 10 base pairs were also considered ineffective due to frequent indels from sequencing errors. A list of targeted genes is provided in Supplementary Table 1.

2.7 Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics version

25.0 (IBM Co., Armonk, NY, USA). Group comparisons were conducted using an independent t-test for continuous variables, and results were reported as means and medians. Categorical variables were analyzed using the χ^2 test or Fisher's exact test, as applicable. Overall and disease-free survival rates were computed using the Kaplan-Meier method and compared via the log-rank test.

3. Result

3.1 Patient Characteristics and Clinical outcome

The cohort comprised 20 patients, including 19 males and 1 female, with a median age of 57. The predominant underlying liver disease was hepatitis B (n= 15, 75%). The median follow-up period was 78.64 months (range 6.27 - 204.73), and the 5-year overall survival rate was 83% after the first operation. The serum concordant group included 7 patients, while the serum discordant group included 13 patients, based on the changes in the serum tumor marker patterns. Figure 2 presents the changes in serum tumor marker patterns for primary and recurrent HCC. No statistically significant differences were found in the composition of tumor marker groups (concordant or discordant) in recurrent tumors based on primary tumor marker patterns (Figure 2, p=0.510).

Patient characteristics were compared between the serum concordant group and the discordant group (Table 1). The mean interval between the 1st and 2nd operations was shorter in the serum concordant group compared to the serum discordant group (Mean 11.16±1.86 vs 44.8±9.45, p<0.001, Figure 3A). The serum concordant group exhibited significantly poorer disease-free survival after the second surgery compared to the discordant group (p=0.039, Figure 3B).

3.2 Genetic Mutation Profile

Genetic analysis was performed on a total of 20 patients (Figure 4). Mutations were detected in 17 patients, identifying a total of 28 mutations, with TP53 being the most common (n=9, 32.14%). Five patients were excluded due to the absence of detectable genetic mutations. Following exclusion, 8 patients were categorized into the genetic concordant group (same mutation present in both primary and recurrent cancer tissues) and 7 into the genetic discordant group (different mutations in both tissues). The mean interval between the 1st and 2nd operations was shorter in the genetic concordant group compared to the genetic discordant group ($p < 0.05$, Figure 5A). The genetic concordant group had significantly worse disease-free survival after the second surgery compared to the discordant group (Figure 5B).

The mutation profiles in primary and secondary tumors were analyzed and summarized based on their serum concordance (Figure 6). In the serum concordant group, most patients were also in the genetic concordant group, while in the serum discordant group, most were in the genetic discordant group. There was a tendency for the concordance of serum tumor markers and genetic profiles to match among the patients ($p < 0.05$, Table 2)

4. Discussion

HCC predominantly arises in cirrhotic livers due to chronic hepatitis, alcoholic liver disease, or NAFLD. The cirrhotic condition significantly increases the risk of HCC, leading to a high recurrence rate in the remnant liver following initial treatment. Recurred tumors exhibit diverse tumor biology, which could indicate both favorable and unfavorable prognoses.(16) Effective treatment strategies for recurrent tumors can be developed by providing personalized therapy based on the biology and prognosis of the tumor. However, accurately predicting the biology of recurrent tumors currently faces significant limitations.

This study reveals that the expression patterns of serum tumor markers can predict the biology and prognosis of recurrent HCC. (Figure 8) The present study found a close association between the concordance and discordance patterns of serum tumor markers in primary HCC and recurrent HCC, and those of gene mutations. This suggests that serum tumor marker patterns may reflect the genetic characteristics of recurrent HCC and assist in predicting its tumor biology. Therefore, identifying the tumor marker pattern of the patient could inform subsequent treatment plans or predict the future course of the patient.

Tumor recurrence after primary treatment can be classified as MO or IM.(14) Multicentric occurrence(MO) is considered as de novo tumor from background liver, and shows more favorable prognosis compared to intrahepatic metastasis(IM), with lower recurrence rates and improved overall survival.(17) Furthermore, multicentric occurrence show better response to additional surgical interventions or locoregional therapies.(15) Although both are classified as recurrent HCC, the prognosis varies between intrahepatic metastasis and multicentric occurrence. Recognizing

these differences, the capability to distinguish between the two types of recurrent HCC is crucial for determining treatment strategies and predicting prognosis. However, no clear standard exists for distinguishing these two entities. While current practice employs a two-year recurrence time point as a criterion, this method is inadequate for accurately identifying the biology of recurrent masses.(12, 13) Although histopathological features such as vascular invasion and tumor differentiation have been proposed as criteria to distinguish recurrent HCC, these methods are not precise, and results cannot be obtained preoperatively. Several studies have employed point mutations analyzed through gene sequencing to assess relatedness, providing a more definitive approach to differentiating recurrent HCC.(18-21) Theoretically, comparing the genetic profiles of primary and recurrent tumors can offer precise insights into tumor biology and the distinction between MO and IM. However, the high costs of these technologies and the considerable time required for results pose significant challenges to their clinical application for all patients. This study shows a correlation between easily measurable serum tumor markers and genetic profiles, suggesting their potential use as an indirect method for predicting the tumor biology of recurrent HCC.

This study revealed that patients with similar serum biomarker and genetic profiles between primary and recurrent tumors experienced early recurrence after initial surgery and exhibited poor prognoses even after subsequent surgeries. These findings suggest that identical serum biomarker patterns in the 1st and 2nd surgeries likely indicate molecular characteristics of intrahepatic metastasis, whereas differing patterns may signify characteristics associated with multicentric occurrences. Therefore, analyzing changes in serum biomarker patterns between primary and recurrent tumors can facilitate the prediction of tumor

recurrence prognosis and aid in tailoring personalized treatment strategies. For instance, in cases predicting poor outcomes, consideration of more aggressive therapeutic strategies, such as radical resection or liver transplantation, may enhance patient outcomes.

Systemic therapy, including the tyrosine kinase inhibitor Sorafenib and developing agents such as Linifanib and Erlotinib, is employed for patients with metastatic HCC when local control is not feasible.(22) However, response rates to these targeted therapies significantly vary due to genetic heterogeneity among HCC subtypes. Predicting which patients will respond favorably to targeted therapies in clinical settings remains challenging. Presently, only 10–20% of patients achieve a good response to Sorafenib.(23) Identifying a patient's genetic profile to foresee responses to Sorafenib is time-consuming and costly, which hinders its routine clinical application. Our study may offer a way to measure tumor marker patterns that could predict favorable genetic mutation profiles for response to this systemic therapy.

This study has several limitations. Subgroup analysis based on tumor marker patterns is constrained by the small number of cases. For example, in instances where both primary and recurrent tumor biomarkers were normal (Group D), it is anticipated that even concordant groups may display a favorable prognosis. However, such detailed comparisons were not feasible in this study. Additionally, the limited number of cases posed challenges for validating the study results. Although NGS was utilized for analysis, the targeted sequencing approach resulted in the omission of some driver gene mutations. Therefore, validation of these findings with a larger patient cohort is necessary.

In conclusion, the expression patterns of serum tumor markers in primary and recurrent HCC have possibility to predict the molecular

biology and prognosis of recurrent HCC. This approach may offer the potential to propose patient-specific therapeutic strategies for recurrent tumors.

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Characteristics of Recurred Hepatocellular Carcinoma Based on Serum Biomarkers and Genetic Mutations

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(Abstract)

This study examines the correlation between serum tumor marker patterns and genetic profiles in both primary and recurrent HCC to better predict tumor biology and patient outcomes. A cohort of 20 patients who underwent surgery for both primary and recurrent HCC was analyzed. Tumor tissues from initial and subsequent surgeries were subjected to genetic mutation analysis using next-generation sequencing (NGS). Based on pre-surgical serum levels of AFP and PIVKA-II, patients were divided into four groups. Patients showing consistent tumor marker expression patterns between primary and recurrent HCC were categorized into the concordant group, while those with varying patterns were placed into the discordant group. The clinical, survival, and mutation profiles between these groups were then compared. The serum discordant group showed significantly improved disease-free

survival (DFS) as opposed to their concordant counterparts. The concordant group patients were significantly more likely to exhibit identical gene mutations across primary and recurrent HCC tissues, whereas the discordant group displayed a variety of mutation patterns in the tissues. The patterns of serum tumor markers in primary and recurrent HCC can predict the molecular biology and prognosis of recurrent HCC effectively. This method provides a basis for proposing patient-specific therapeutic approaches for recurrent tumors.

재발된 간암의 혈액 종양 표지자와 유전자형에 따른 특성 규명

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(초록)

본 연구는 원발 및 재발 간세포암(HCC)에서 혈청 종양 표지자 패턴과 유전자 프로파일 간의 상관관계를 분석하여 종양 생물학적 특성과 환자의 예후를 보다 정확히 예측하고자 하였다. 원발 및 재발 HCC로 수술을 시행받은 20명의 환자군을 분석 대상으로 하였다. 초회 및 재수술에서 얻은 종양 조직은 차세대 염기서열 분석(NGS)을 통해 유전자 돌연변이 분석을 수행하였다. 수술 전 혈청 AFP와 PIVKA-II 수치를 기준으로 환자들을 네 그룹으로 나누었다. 원발 및 재발 HCC 사이에 종양 표지자 발현 패턴이 일치하는 환자들은 일치군(concordant group)으로, 패턴이 달라진 환자들은 불일치군(discordant group)으로 분류하였다. 이후 각 그룹 간의 임상적 특성, 생존율 및 돌연변이 프로파일을 비교 분석하였다. 혈청 불일치군은 일치군에 비해 유의하게 개선된 무병생존율(DFS)을 보였다. 일치군의 경우 원발 및 재발 HCC 조직에서 동일한 유전자 돌연변이가 관찰될 가능성이 더 높았던 반면, 불일치군에서는 다양한 돌연변이 패턴이 나타났다. 원발

및 재발 HCC에서의 혈청 종양 표지자 패턴은 재발 종양의 분자생물학적 특성과 예후를 효과적으로 예측할 수 있으며, 이를 통해 환자 맞춤형 재발 종양 치료 전략을 제시할 수 있는 근거를 제공한다.

Table 1. Patient Characteristics

Characteristics (Tumor Marker)	Concordant (n=7)	Discordant (n=13)	p-value
Sex, n (%)			
Female/Male	0/7(100)	1(7.7)/12(92.3)	>0.05
Age, mean (SD), yr	59.1(10.32)	56.85 (6.20)	>0.05
Body mass index, mean (SD), kg/m ²	24.42(3.37)	24.41(1.93)	>0.05
1 st AFP(ng/mL) (median,range)	12.4(2.19-7069.1)	26.8(1.06-22609)	>0.05
2 nd AFP(ng/mL) (median,range)	5.3(1.3-10684)	23.7(2.2-18133.1)	>0.05
1 st PIVKA-II(ng/mL) (median, range)	54(26.74-509.3)	150.6(24-4302)	>0.05
2 nd PIVKA-II(ng/mL) (median,range)	44(16.7-6881)	68.1(19.87-8656)	>0.05
Risk factors, n(%)			
Hepatitis B	4(57.1)	11(84.6)	>0.05
Hepatitis C	1(14.3)	1(7.7)	
Alcohol	1(14.3)	1(7.7)	
Fatty liver (NAFLD)	1(14.3)	0	
Tumor size(Primary) (median, range)	3.7(1.3-6)	3(1.7-9)	>0.05
Vascular invasion(Primary), n(%)	2(28.6)	3(23.1)	>0.05
Tumor size(Recurrent) (median, range)	3(1.1-16.7)	2.5(1.5-7)	>0.05
Vascular invasion(Recurrent), n(%)	2(28.6)	4(30.8)	>0.05

Table 2. Diagnostic Significance of Tumor Marker-Based Decision

		Genetic Profile	
		Concordant (n=8)	Discordant (n=7)
Tumor marker	Concordant (n=7)	7	0
	Discordant (n=8)	1	7

Figure 1. Group by Tumor Marker Traits



Figure 2. Serum Tumor Marker Pattern in Primary and Recurrent Tumor

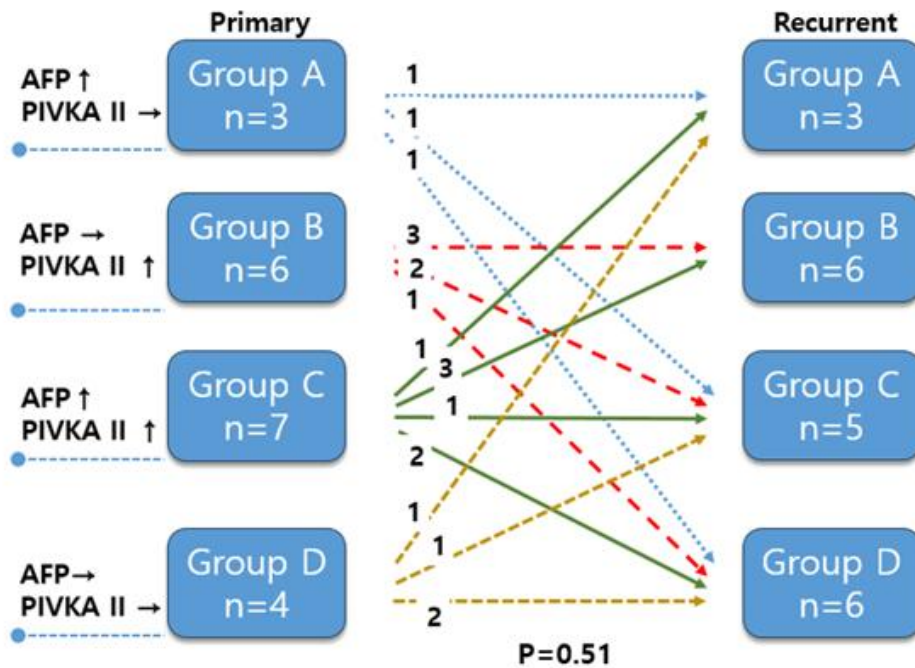


Figure 3. Survival Analysis Based on Serum Tumor Marker Patterns

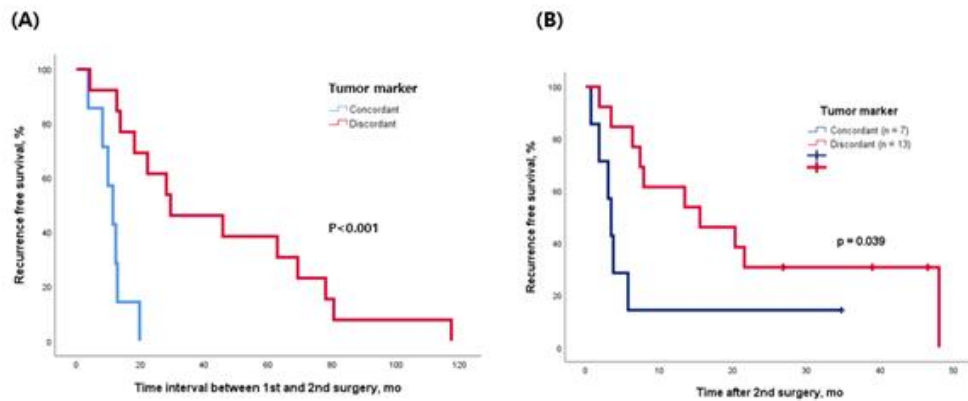


Figure 4. Mutation Profile

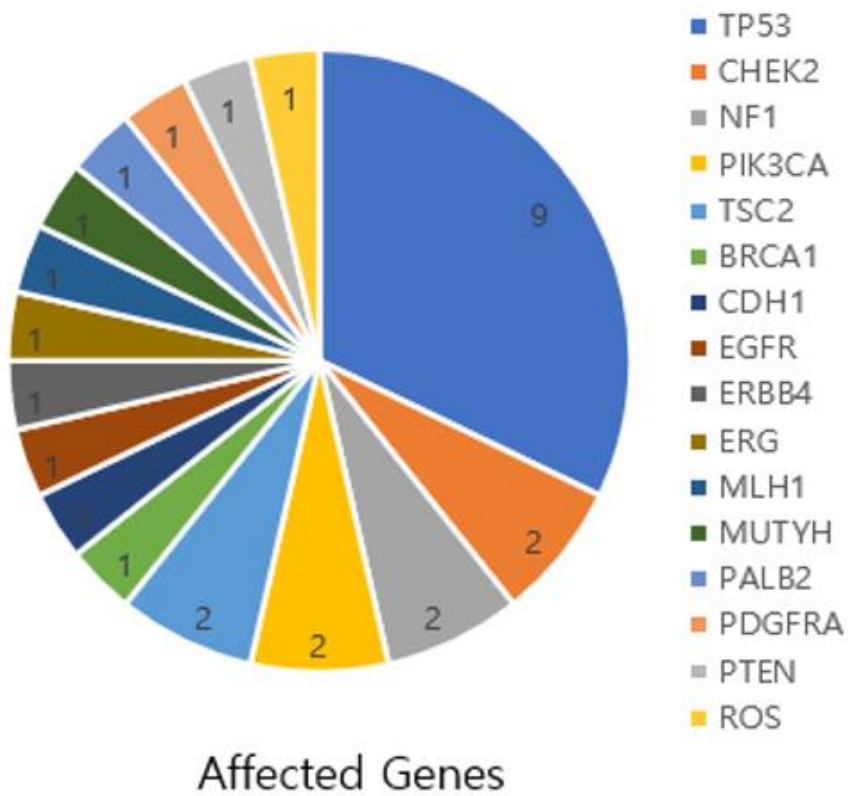


Figure 5. Survival Analysis Based on Mutation Profile

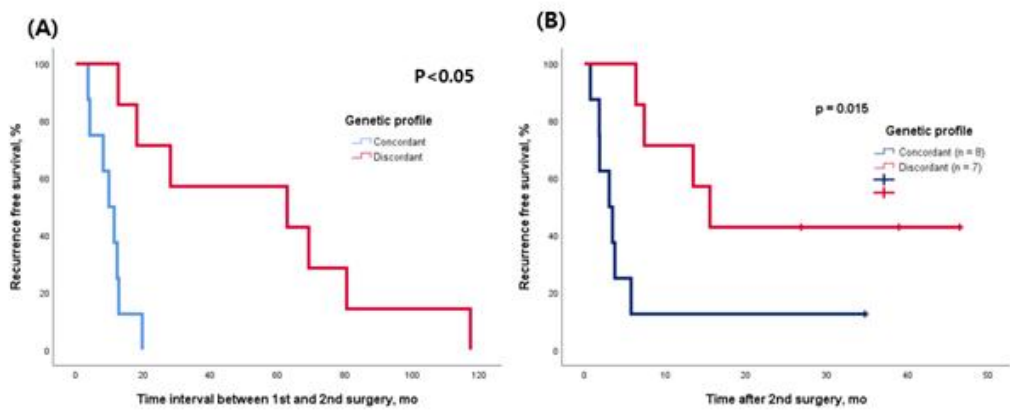


Figure 6. Genetic Profile Sorted by Serum Group

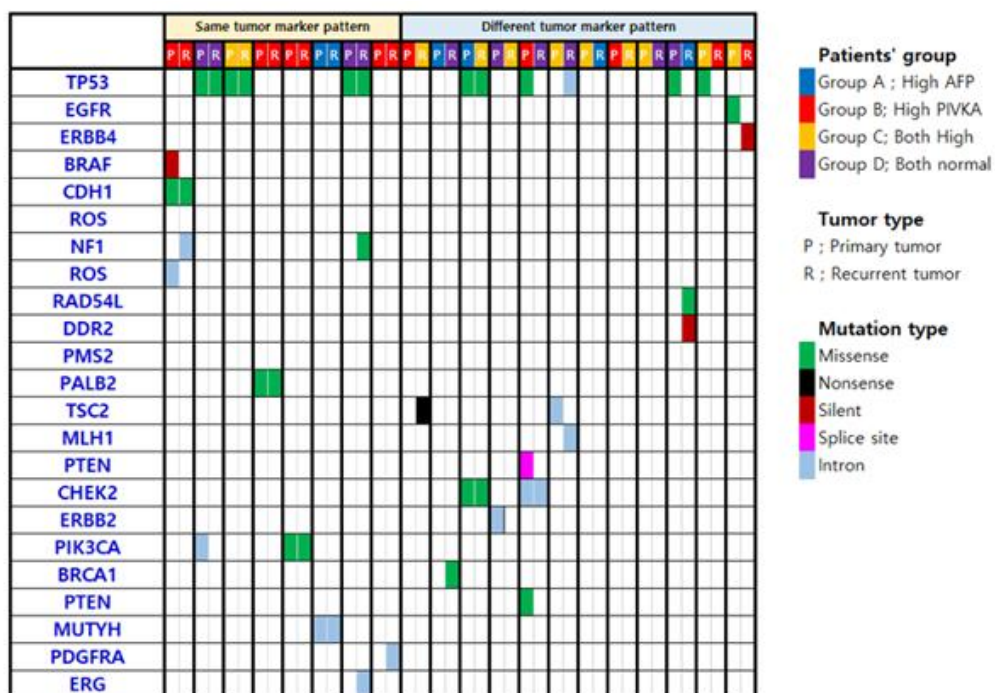
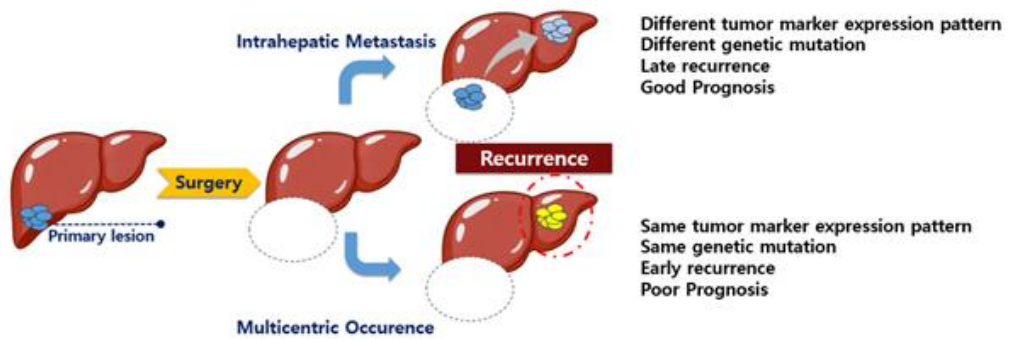


Figure 8. Schematics of Hypothesis



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