Original paper

# Short-term prognosis of childhood hepatoblastoma in relation to *ERCC1 C118T* single nucleotide polymorphism and VEGF expression

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To evaluate the short-term prognosis of pediatric hepatoblastoma (HB) in relation to excision repair cross-complementation gene 1 (*ERCC1*) C118T single nucleotide polymorphism (SNP) and VEGF expression.

*ERCC1* C118T SNP and VEGF expression were detected and investigated in 31 children with HB undergoing platinum-based chemotherapy, to analyze their relationship with short-term pediatric HB prognosis.

CC (38.7%; 12/31), CT (35.5%; 11/31), and TT (25.8%; 8/31) *ERCC1* C118T mutation types were identified. The Kaplan-Meier survival curve analysis showed that the CC group had a better short-term prognosis than the CT + TT group (p = 0.010). VEGF was overexpressed in 14 cases (45.2%) and underexpressed in 17 cases (54.8%). The Kaplan-Meier survival curve analysis showed that the high VEGF expression group showed poorer short-term prognosis than the lower VEGF expression group (p = 0.004).

In this study, *ERCC1* C118T SNP in children with HB was mainly found to be mutant type CT + TT. Compared to wild type CC, children with the mutant type CT + TT exhibited better treatment efficacy and remission with platinum-based chemotherapy as well as better survival rates. Moreover, the short-term prognosis of children with low VEGF expression was better than in those with high expression.

**Key words:** *ERCC1* gene, single nucleotide polymorphism, VEGF, hepatoblastoma, prognosis, platinum drugs.

## Introduction

Hepatoblastoma (HB) is a rare malignant tumor in children, accounting for less than 1% of all their malignancies. However, it remains the most common form of pediatric liver malignancy [1], the incidence rate of which is about 1.2-1.5 out of every million [2].

Before the early 1970s, HB treatment relied mainly on surgery, and the survival rate was only 20-30%. With the introduction of platinum-based chemotherapeutic drugs (PDs), the post-treatment prognosis of HB has been greatly improved, and the 3-year overall survival rate can reach up to 60-70% [3, 4]. Platinum-based chemotherapeutic drugs are the main drugs used against HB. Almost all HB cases require preoperative and/or postoperative chemotherapy [5]. It has been reported that if well-differentiated PRETEXT Stage I epithelial HB can be completely resected, no postoperative chemotherapy

is required, prognosis is good, and the survival rate can be 100% [6].

*ERCC1* (excision repair cross-complementation gene 1) was the first DNA damage repair gene to be discovered in humans. It participates in the nucleotide excision repair (NER) process, which is an important mechanism in the resistance to PDs [7]. *ERCC1* single nucleotide polymorphisms (SNPs), particularly the C-T mutation at the 118th codon in exon 4, can affect gene expression level and change gene copy number, thus affecting the resistance to PDs [8]. Many studies have reported that the *ERCC1* C118T mutation exhibits a predictive value for PD efficacy and disease prognosis in different types of cancers, such as non-small cell lung cancer [8], ovarian cancer [9], gastric cancer [10], esophageal cancer [11], pancreatic cancer [12], and colorectal cancer [13]. Therefore, screening *ERCC1* mutation before chemotherapy may have important guiding significance and prognostic value for chemotherapy.

Vascular endothelial growth factor (VEGF) is a highly-specific vascular endothelial cell mitogen that promotes endothelial cell proliferation, angiogenesis, and vascular permeability. It is the most important growth factor for angiogenesis [14]. Studies have shown that VEGF expression is closely related to angiogenesis, growth, invasion, and metastasis in tumors. High VEGF expression in liver tumors and childhood HB is also associated with poor prognosis [15, 16].

Since *ERCC1*-C118T mutation studies are rare and reported VEGF cases are limited in childhood HB, we used a retrospective cohort study to investigate the short-term prognosis of HB in children in relation to *ERCC1* C118T mutation and VEGF expression. Here, *ERCC1* C118T mutation and VEGF expression were detected in 31 children with HB, and the effects of chemotherapy and short-term prognosis were reported for relation-based analysis and discussion.

## Materials and methods

### Clinical data

From January 2013 to June 2016, 45 children with HB (pathologically diagnosed and undergoing surgery) were treated at the Department of Pediatrics, Beijing Tongren Hospital, Capital Medical University. Among them, 31 children were tested for the *ERCC1* C118T SNP using their blood samples and for VEGF expression from surgical samples of their tumors. The rest 14 patients were excluded due to refusal to sign informed consent. The children were staged according to the postoperative HB staging criteria developed by the Children's Oncology Group (COG; USA), and started on platinum-based chemotherapy regimens and follow-up routines. The follow-up endpoints were set either as relapse during follow-up/death or end of the 2-year follow-up. No case was lost during follow-up. This study was conducted in accordance with the declaration of Helsinki. This study was conducted with approval from the Ethics Committee of Capital Medical University. Written informed consent was obtained from all participants' guardians.

### Diagnostic and staging criteria

Surgical specimens were obtained from the 31 children to confirm HB via pathological examination. The pathological classification criteria were based on the pathological types proposed by COG in 2014 [17], and all patients were classified based on the COG staging criteria (Table I) [18].

### Detection of C118T mutation

Anticoagulant-containing peripheral blood samples (2 ml) were taken from each child for DNA extraction using magnetic beads. DNA was extracted based on the instructions obtained from the Lab-Aid DNA Separation Kit (Zeesan Biotech Co. Ltd. Xiamen, China). DNA sequencing and TaqMan probing were used for detection and analysis.

### DNA sequencing method

The *ERCC1* primer sequences were as follows: F: GACCACAGGACACGCAGAC and R: TGAGGAACAGGGCACAGG. The amplified target gene fragment was a 3,162-3,595 bp long segmented sequence from the complete *ERCC1* sequence. After amplification, the ABI 3730xL DNA sequencer (Hitachi, USA) was used for gene sequencing.

The TaqMan SNP Genotyping Assays Probe Kit (ABI) was customized to label VIC/FAM according to the human rs11615 (ASN118ASN; base sequence AAC118AAT). The Roche LightCycler® 480 System was used to run PCR and the parameters were as follows: 50°C, 2 min; 95°C, 10 min; 95°C, 15 s; and 60°C, 1 min. The VIC and FAM fluorescent signals were then recorded and the Roche 480 SNP analysis software was used to determine whether the *ERCC1* AAC118AAT site was homozygous or heterozygous.

**Table I.** Postoperative COG Staging criteria of HB

| STAGING | DEFINITION |
|---|---|
| Stage I | Signifies negative surgical margins |
| Stage II | Has residual microscopic disease at the margins |
| Stage III | Represents grossly positive margins or regional lymph node involvement |
| Stage IV | Indicates metastatic disease |

## Detection of VEGF

Immunohistochemical staining was used to detect VEGF expression in the surgical/liver biopsy specimens. The human VEGF immunohistochemical staining kit (Yaji Co. Ltd., Shanghai, China) was used as per the manufacturer's instructions. The general steps were as follows: paraffin embedding, tissue slicing (3 $\mu$m thickness), slice baking, dewaxing, hydration, antigen retrieval, sequential incubation and washing with primary and secondary antibodies, coloration with DBA solution, dehydration, hyalinization, mounting, and observation. The positive control was obtained from Yaji Co. Ltd., and the blank control involved washing with PBS solution in place of the primary antibodies.

Currently, no standard methodologies exist to judge VEGF immunohistochemical staining results in children with HB. After referring to other studies and comprehensively considering the strength of staining and the number of colored cells in this study, yellow- or brown-yellow-stained granules in the cytoplasm were defined positive cell staining. All the slices were observed under a 400× microscope with 5 randomly selected fields from each slice. The proportion of positively-stained cells in the fields was averaged and scored as follows: proportion ≤ 5%, 0 points; 6-25%, 1 point; 26-50%, 2 points; 51-75%, 3 points; and ≥ 75%, 4 points; for staining intensity: no stain, 0 points; light yellow, 1 point; yellow, 2 points; and brown, 3 points. The total score was then calculated as a product of positive cell proportion and staining intensity giving the following results: 0-4 points, low expression; and 5-12 points, high expression [19].

## Efficacy analysis and follow-up

Treatment efficacy was determined using the following parameters based on the RECIST 1.1 evaluation criteria [20]:

1. Complete response (CR): Disappearance of all target lesions. Any pathological lymph nodes (whether target or non-target) must have their short axis reduced to < 10 mm.
2. Partial response (PR): At least a 30% decrease in the sum of the diameters of the target lesions, using the baseline sum diameters as a reference.
3. Progressive disease (PD): At least a 20% increase in the sum of the diameters of the target lesions,

using the smallest sum value in the study as a reference (this includes the baseline sum if it is the smallest sum value in the study). In addition to the 20% relative increase, the sum value must also demonstrate an absolute increase of at least 5 mm. The appearance of one or more new lesions is also considered to be progression.

4. Stable disease (SD): Neither sufficient shrinkage to qualify for PR, nor sufficient increase to qualify for PD, using the smallest sum diameter value in the study as a reference.

Follow-up started at the end of chemotherapy and lasted for 2 years. The end point was decided as recurrence/death during the follow-up or completion of the 2-year follow-up.

At the end of the study, since there were no SD patients, the CR and PR cases were categorized into the remission group (Group R), and the PD, relapse, and death cases were categorized into the non-remission group (Group NR). Group R was considered to be progression-free survival (PFS) cases [20].

## Statistical analysis

The SPSS 20.0 software was used for statistical analysis. Count data was determined using Fisher's exact test or continuous calibration $\chi^2$ test. Survival analysis was performed using the Kaplan-Meier method, and $p < 0.05$ was considered to be statistically significant.

## Results

### General information

Thirty-one cases were included in the study. Diagnosis age ranged from 1 month to 156 months, with the average being 34.23 ±34.89 months. The general information is shown in Table II. The number of male and female patients were 22 and 9, respectively. There were 2 stage II, 12 stage III, and 17 stage IV cases. The pathological types included 20 epithelial HB and 11 mixed HB cases. The α-fetoprotein (AFP) levels in all the patients were more than 1000 ng/ml.

### Chemotherapy drugs and follow-up

All 31 patients underwent chemotherapy regimens of platinum-based drugs combined with other drugs.

**Table II.** General information of 31 enrolled children

|  | M | F | Stage II | Stage III | Stage IV | Epithelial HB | Mixed HB |
|---|---|---|---|---|---|---|---|
| n | 22 | 9 | 2 | 12 | 17 | 20 | 11 |
| Ratio | 71.0% | 29.0% | 6.5% | 38.7% | 54.8% | 64.5% | 35.5% |

The patients were divided into low-risk, intermediate-risk, and high-risk based on the COG risk stratification [21]. Platinum-based C5V (low risk) and C5VD (intermediate and high risk) treatments were used for the different risk stratifications [21]. The C5V regimen consisted of cisplatin (90 mg/m², > 1 year old; or 3 mg/kg, < 1 year old) on day 1, and vincristine (1.5 mg/m² with a maximum dose of 2 mg) and 5-fluorouracil (600 mg/m²) on day 2. The C5VD regimen consisted of cisplatin (90 mg/m², > 1 year old; or 3 mg/kg, < 1 year old) on day 1, vincristine (1.5 mg/m² with a maximum dose of 2 mg) and 5-fluorouracil (600 mg/m²)

A



B



C



**Fig. 1.** Examples of ERCC1 gene C118T mutation: A) CC wild homozygote; B) CT mutant heterozygote; C) TT mutant homozygote

on day 2, and doxorubicin (25 mg/(m² · d)) on days 2 and 3.

At the end of the follow-up, 38.7% of the patients (12/31) achieved CR, 19.4% (6/31) achieved PR, 9.7% (3/31) achieved PD, 19.4% (6/31) relapsed, and 12.9% (4/31) died. Group R accounted for 58.1% (18/31) of the patients and Group NR accounted for 41.9% (13/31).

### Detection of C118T mutation

The analysis of the *ERCC1* C118T mutation in the 31 patients detected 38.7% (12/31) as CC wild homozygote, 35.5% (11/31) as CT mutant heterozygote, and 25.8% (8/31) as TT mutant homozygote. Mutant type CT + TT accounted for 61.3% of all patients, and wild type CC accounted for 38.7% of all patients. The results of the gene sequencing are shown in Fig. 1 (the arrow shows the *ERCC1* SNP location on exon 4 codon 118). Statistical significance of deviations from the Hardy-Weinberg equilibrium was determined using the Pearson's chi-square test ($\chi^2$ = 1.083, p = 0.582), which meant that the distribution of the C118T polymorphism was within the Hardy-Weinberg equilibrium.

### VEGF test results

VEGF detection in the pathological specimens of the 31 patients showed high VEGF expression in 45.2% (14/31) and low VEGF expression in 54.8% (17/31) of the specimens.

### Relationship between clinical stage and VEGF expression

Patients were divided into the II + III stage group (Group II + III) and the IV stage group (Group IV) based on the HB clinical staging, and into the high expression (Group H) and low expression (Group L) groups using Fisher's exact test based on the VEGF detection results (Table III). The ratio of high VEGF expression in Group II + III was observed to be 3/14 and Group IV was 11/17. The difference was statistically significant (p = 0.029). Moreover, the high VEGF expression rate in Group II + III was lower than that of Group IV.

### Relationship between VEGF expression and prognosis

The results of the Fisher's exact test after the 2-year follow-up revealed that PFS proportion was 28.6% in Group H (4/14) and 82.4% in Group L (14/17). The difference was statistically significant (p = 0.004; Table IV).

The results of the Kaplan-Meier survival analysis (Fig. 2) suggested that the PFS status in Group L was better than that in Group H. The difference was statistically significant (p = 0.005).
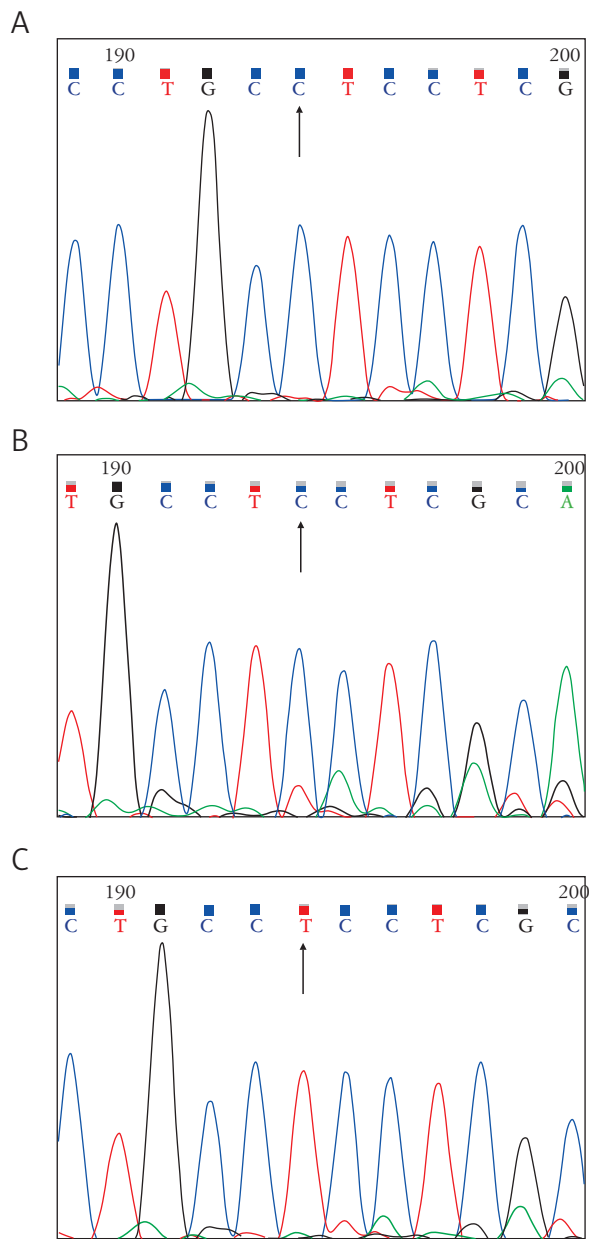
**Table III.** Relationship between clinical stage and VEGF expression in HB

|  | GROUP II + III | GROUP IV | SUM |
|---|---|---|---|
| Group H | 3 | 11 | 14 |
| Group L | 11 | 6 | 17 |
| Sum | 14 | 17 | 31 |

**Table IV.** VEGF expression and prognosis of HB

|  | GROUP H | GROUP L | SUM |
|---|---|---|---|
| Group R | 4 | 14 | 18 |
| Group R/D | 10 | 3 | 13 |
| Sum | 14 | 17 | 31 |

**Table V.** ERCC1 C118T types with prognosis of children with HB

|  | TT + CT | CC | SUM |
|---|---|---|---|
| Group R | 16 | 2 | 18 |
| Group R/D | 5 | 8 | 13 |
| Sum | 21 | 10 | 31 |

## Relationship between the ERCC1 C118T types and prognosis

Patients were divided into mutant TT + CT and wild CC groups based on the *ERCC1* C118T types. The continuous calibration chi-square test (Table V) suggested that the 2-year PFS ratio was 76.2% in group TT + CT (16/21) and 20% in group CC (2/10). The difference was statistically significant ($p = 0.010$) i.e., the post-treatment PFS rate in Group TT + CT was higher than that in Group CC. The Kaplan-Meier analysis revealed that the survival condition for the 2-year PFS in group TT + CT was better than that in group CC (Fig. 3). The difference was statistically significant ($p = 0.008$).

## Discussion

Currently, HB therapeutic strategies mainly consist of surgery and chemotherapy. The surgical removal of lesions has a crucial influence on prognosis [22]. In children who cannot undergo complete resection, prognosis mainly depends on chemotherapy. While preoperative adjuvant chemotherapy can increase the rate of surgical resection, Postoperative chemotherapy is of great significance in eliminating residual lesions [23, 24]. The application of PDs in the 1970s significantly increased the prognosis of HB treatments. Currently, cisplatin alone (low-dose chemotherapy regimen) for low-risk HB, PLADO and C5V for standard-risk HB, and Super
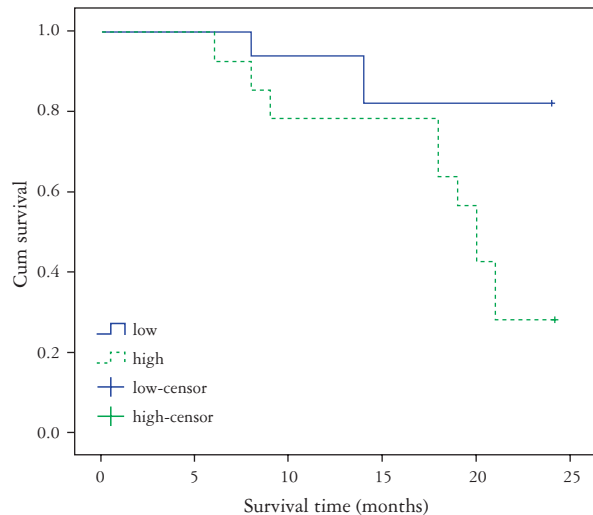


**Fig. 2.** Kaplan-Meier 2-year progression free survival analysis of VEGF high expression group and low expression group
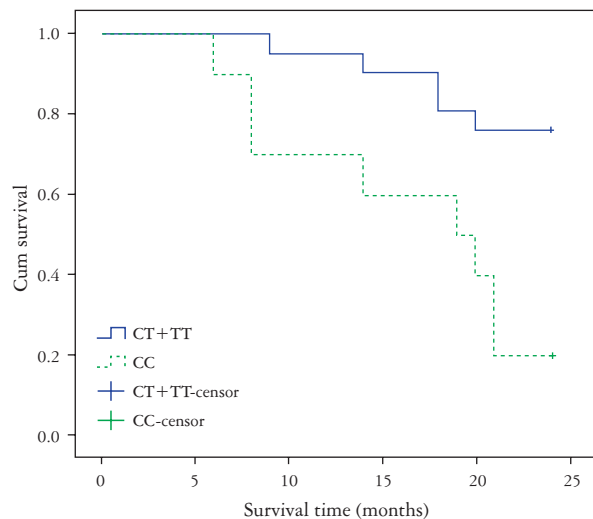


**Fig. 3.** Kaplan-Meier 2-year progression free survival analysis of ERCC1 gene CC group and CT+TT group

PLADO and C5VD for high-risk HB are the main, indispensable platinum drugs used for treatment [22, 25, 26]. After entering the body, the platinum drugs bind to DNA to form intra-strand and inter-strand crosslinks, which induces DNA damage due to this covalent DNA binding, thereby inhibiting DNA synthesis and replication, causing cell death, and suppressing tumor cell growth [27]. DNA damage correction caused by platinum drugs *in vivo* mainly depends on the nucleotide excision/repair mechanism, which specifically removes the platinum-DNA adducts, repairs DNA damage, and causes PD resistance [28].

*ERCC1* was the first DNA damage repair gene to be discovered in humans, and its expressed product participates in DNA strand cleavage and damage

repair via the NER pathway [29]. It is the key rate-limiting enzyme in the nucleotide excision/repair mechanism [30]. A common and significant *ERCC1* SNP is the C→T mutation at codon 118 on exon 4, causing the codon AAC to become AAT [13]. This is a nonsense mutation, which could consist of a C/C wild homozygote, T/T mutant homozygote, or C/T mutant heterozygote. There is no change in the encoded aspartic acid, but the usage frequency of the AAT codon is lower than that of AAC, resulting in the decreased expression of the mutant *ERCC1* gene carrying the T base [31]. Higher *ERCC1* expression levels are associated with stronger NER abilities and higher resistance to platinum drugs [32]. C118T mutation reduces *ERCC1* expression, NER, and the ability of *ERCC1* to repair DNA damage caused by platinum. Patients with the T allele may also be more sensitive to PDs [31, 32]. Globally, many studies in non-small cell lung cancer [8], ovarian cancer [9], gastric cancer [10], esophageal cancer [11], pancreatic cancer [12], and colorectal cancer [13] have shown patients with the CT or TT genotypes in *ERCC1* C118T mutation to be more sensitive to PDs when compared to patients with the CC genotype.

In this study, the *ERCC1* C118T SNP in the 31 children with HB was mainly found to be the CT + TT (19/31) mutation (61.3% of the children exhibited the C → T mutation). This study further analyzed the correlation between the *ERCC1* genotype and relapse/remission. The results showed that the remission rate in children with CT + TT was higher than those with CC. The 2-year survival analysis showed that the survival condition of group CT + TT was better than that of group CC i.e., the 2-year remission rate was high and survival condition was better. It is not difficult to infer that the children with CT + TT had a reduced ability to repair nucleotide excision; therefore, they had a lower ability to repair PD-induced damage, were more sensitive to PDs, and exhibited better chemotherapy results. The above inference is consistent with the reports of other studies both in and outside China [8-13].

VEGF is a highly-specific vascular endothelial cell mitogen that promotes vascular endothelial cell proliferation, angiogenesis, and increases vascular permeability [14]. It is the most important cytokine in tumor angiogenesis. VEGF is closely related to the growth, invasion, metastasis, and prognosis of tumors [15, 16]. In recent years, studies have shown that pediatric liver malignancies have the characteristics of wide-range angiogenesis, and new blood vessels cause rapid tumor growth and lead to poor prognosis [16].

This study showed that VEGF expression rate was significantly higher in clinical stage IV patients (distant tumor metastasis) when compared to clinical stage II + III patients. This suggested that VEGF may play certain roles in tumor invasion and metastasis. Moreover, the 2-year remission rate was significantly higher and survival was better in children with low VEGF expression when compared to those with high VEGF expression. This suggested that VEGF had a certain predictive effect on the prognosis of such children. Recently, experiments have shown that the application of the VEGF antibody can significantly inhibit the angiogenesis and tumor growth of human HB cells cultured *in vitro* [33]. VEGF-targeted drugs, such as the VEGF monoclonal antibody bevacizumab and the VEGF receptor-selective tyrosine kinase inhibitor sorafenib, developed for malignant tumors and approved by the FDA, have demonstrated efficacy in treating kidney and liver cancers [34, 35]. Bevacizumab combined with sorafenib was also reported to stabilize HB and reduce α-fetoprotein levels in a patient suffering from recurrent HB and distant metastasis [36]. Combined with the results from the present study, results showing patients with distant HB metastasis (stage IV) having high VEGF expression and patients with high VEGF expression having low 2-year chemotherapy remission rates suggested VEGF-targeted treatments as good alternative treatment options against HB in children with high VEGF expression.

In this study, *ERCC1* C118T SNP in children with HB was mainly found to consist of the CT + TT mutant type. Children with T allele mutations had better treatment efficacy and higher 2-year PFS rates. Moreover, stage IV patients were found to have high VEGF expression rates and consequently, low 2-year PFS rates.

However, this study was limited as it was a single-center trial. Additionally, only a few cases were included and the study time was short. In order to analyze medium/long-term survival conditions, multi-center trials, larger sample sizes, and longer study times are required.

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