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Mutational signature, cancer driver genes mutations and transcriptomic subgroups predict hepatoblastoma survival

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ABSTRACT

Background: Hepatoblastoma is the most frequent pediatric liver cancer. The current treatments lead to 80% of survival rate at 5 years. In this study, we evaluated the clinical relevance of molecular features to identify patients at risk of chemoresistance, relapse and death of disease.

Methods: All the clinical data of 86 children with hepatoblastoma were retrospectively collected. Pathological slides were reviewed, tumor DNA sequencing (by whole exome, whole genome or target) and transcriptomic profiling with RNAseq or 300-genes panel were performed. Associations between the clinical, pathological, mutational and transcriptomic data were investigated.

Results: High-risk patients represented 44% of our series and the median age at diagnosis was 21.9 months (range: 0–208). Alterations of the WNT/ß-catenin pathway and of the 11p15.5 imprinted locus were identified in 98% and 74% of the tumors, respectively. Other cancer driver genes mutations were only found in less than 11% of tumors. After neoadjuvant chemotherapy, disease-specific survival and poor response to neoadjuvant chemotherapy were associated with 'Liver Progenitor' ($p = 0.00049$, $p < 0.0001$) and 'Immune Cold' ($p = 0.0011$, $p < 0.0001$) transcriptomic tumor subtypes, SBS35 cisplatin mutational signature ($p = 0.018$, $p = 0.001$), mutations in rare cancer driver genes ($p = 0.0039$, $p = 0.0017$) and embryonal predominant histological type ($p = 0.0013$, $p = 0.0077$), respectively. Integration of the clinical and molecular features revealed a cluster

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of molecular markers associated with resistance to chemotherapy and survival, enlightening transcriptomic 'Immune Cold' and Liver Progenitor' as a predictor of survival independent of the clinical features.
Conclusions: Response to neoadjuvant chemotherapy and survival in children treated for hepatoblastoma are associated with genomic and pathological features independently of the clinical features.

1. Introduction

Hepatoblastoma (HB) is the most frequent pediatric liver cancer, representing around 1–1.5 cases per million children[1]. In the last decades, following the introduction of cisplatin-based chemotherapy and the use of prognostic factors in the risk stratification to determine the most adapted treatment, the survival of children with HB has improved considerably[2–8]. Currently, the Children's Hepatic tumors International Collaboration (CHIC) scoring based on the radiological PRETEXT (PRETreatment EXTent disease) staging, age, presence of metastases, and alpha-fetoprotein serum level (AFP) at diagnosis is the most widely used risk stratification system, in particular in the PHITT international clinical trial[3,9]. Moreover, tumor histological characteristics and genetic markers have been described associated to HB prognosis, but they are not currently used in clinical setting because they remain to be validated[4,10–12]. Nowadays, the overall 5-year survival of patients with standard-risk HB is around 90%, and 65% for high-risk HB[3,5]. However, some patients develop primary or secondary chemoresistance and there are currently no consensual therapeutic alternatives for targeting resistant HB, enlightening the urgent need to identify additional prognostic factors based on histology or molecular features[10,11,13]. HB demonstrate an important phenotypic pathological diversity with more than 10 described histological subtypes and high intra-tumor heterogeneity. Epithelial HB may exhibit several patterns (alone or in combination): fetal, embryonal, small cell undifferentiated, cholangioblastic and macrotrabecular. The mixed mesenchymal–epithelial HB subtype is characterized by the presence of mature and immature fibrous tissue, chondroid and osteoid components, as well as of teratoid features (endodermal, neuroectodermal tissue and striated muscle) that account for the embryonal origin of this tumor[14,15]. At the genomic level, HB are characterized by a very low number of somatic coding mutations and only a few cancer driver genes with recurrent alterations[16–18]. β-catenin is the oncogene most frequently activated by mutations in *CTNNB1* (~90% of HB) or *APC* (~5%) genes. Oncogenic Insulin Growth Factor type 2 (*IGF2*) over-expression is the second hallmark of HB due to 11p15.5 alteration in ~80% of tumors, including 17% of the patients with pre-neoplastic mosaic expansions in the liver [17,19,20]. In contrast, recurrent alterations in *TERT* (~10%) are identified mainly in old children with HB, and their association with poor prognosis remains to be validated [10,11,16,17,19,21].

Despite this relative genomic simplicity, three major transcriptomic HB sub-classes have been described: **(1)** well-differentiated tumors related to fetal histology (Cairo-Hooks 'C' / Hirsch 'Hepatocytic' / Nagae 'Hepatocyte'), **(2)** proliferative progenitor HB associated to embryonal histology (Cairo-Hooks 'C2A' / Hirsch 'Liver Progenitor' / Nagae 'Proliferative') and **(3)** HB with mesenchymal differentiation (Hooks 'C2B' / Hirsch 'Mesenchymal' / Nagae 'Mesenchymal')[17,19,22]. Of note, hepatocytic HBs are related to high tumor immune infiltration after chemotherapy[17]. In contrast, the 'Liver Progenitor' /proliferative tumors are able to proliferate under chemotherapy and they are at the origin of relapses and metastases development. This has been demonstrated by the subclonal accumulation of cisplatin-induced mutations (SBS35 mutational signature) in primary tumors that are retrieved clonally in HB relapses and metastases[17]. The analysis of multiple samples or at the unique cell level also highlighted the molecular heterogeneity and cell plasticity in HB [17,23–25].

Even though several associations between molecular features, transcriptomic classes and the outcome of HB patients have been described, currently, there are still no molecular or histological markers included in the therapeutic stratification[10,11,16,26–28]. This is partly due to the rarity of the disease, the good overall prognosis and the difficulties to validate in prospective settings. Understanding molecular mechanisms at the origin of chemoresistance may help to identify patients most at risk of relapse and metastasis and help to adapt adjuvant therapy decisions.

We aim to evaluate the clinical relevance of the newly discovered genomic features in driver gene alterations, cisplatin mutational signature and transcriptomic classifications to identify patients most at risk of chemoresistance, relapse and poor survival. In this retrospective study, all the patients treated for an HB between 1991–2021 with available (1) clinical data with a minimal follow-up of one year, (2) DNA genomic analyses (WES, WGS or targeted sequencing) and/or transcriptomic analyses (RNAseq or qRT-PCR gene expression data genes) of the primary tumor, (3) written informed consent have been included.

2. Results

2.1. Clinical features and clinical prognostic factors in hepatoblastoma cohort

In this study, 86 patients with HB were included (sex-ratio M/F=1.9, median age at diagnosis=21.9 months, range=0–208.4 months including 28 patients aged >=3y). Thirteen patients had PRETEXT IV tumors and 23 patients had a metastatic disease at diagnosis (see Table 1 and **Supp.** Table S1 for full description). According to the CHIC stratification, 44% of the patients had a high-risk HB (n = 38/86). Most of the patients (n = 80/86, 93%) received neoadjuvant chemotherapy according to the SIOPEL I to VI or PHITT protocols and the risk stratification followed by surgical resection and adjuvant chemotherapy (**Supp.** Table S1). Patients with CHIC high, intermediate or low-risk received neoadjuvant intensive polychemotherapy in 31/35, 8/10, and 8/35, respectively; the other neoadjuvant chemotherapy was based on cisplatin alone. Six patients with localized PRETEXT I-II tumors had surgery first, including two treated with surgery only. Among the 80 patients treated with neoadjuvant chemotherapy, 17/79 were 'Poor-Responders' according to the clinical definition in supplement material and methods with AFP decrease < 1 log and/or AFP re-ascension (#5043, #5373, #5377, #3081, #3086, #3370, #3549, #3660, #3944, #4001) during the neoadjuvant chemotherapy (**Supp.** Fig. S1). Among these 'Poor-Responders', 4 patients had progressive disease at imaging during neoadjuvant chemotherapy (#3549, #5233, #3676), including one patient (#5377) who died of tumor progression before surgery. Conversely all the 'Good Responders' showed a shrinkage of tumor volume ≥ 30% (RECIST) and a decrease of AFP ≥ 1 log10 without re-ascension of AFP at 2 consecutive times with a minimum of one week in-between and no sign of progressive disease at imaging (lesion increasing and/or new lesion)[9,29].

At the end of the treatment (chemotherapies and surgeries), 88% of the patients were in complete remission (n = 73/83), including 9 patients 'Poor-Responders' to neoadjuvant chemotherapy. Among the 10 patients with residual disease at the end of first-line treatment, 8 were already 'Poor-Responders' to the neoadjuvant chemotherapy ($p < 0.0001$). After a median follow-up of 63 months (range: 7–283 months), progression was observed in 10 patients (4 during neoadjuvant chemotherapy and 6 after adjuvant chemotherapy), 18 patients relapsed (including one relapse 13 years after the initial diagnosis), and 14 patients died of progressive disease. Moreover, one patient in complete remission of his HB died of acute myeloid leukemia 42 months after the

Table 1
Demographic and clinical data of hepatoblastoma series (n=86).

		Number of analyzed patients	Number (%)/Median (min-max)
Age at diagnosis (months)		86	21.9 (0-208.4)
< 3 years			58 (67.4%)
3 - 8 years			16 (18.6%)
≥ 8 years			12 (14%)
Gender (Male)		86	56 (65.1%)
Predisposition		86	
FAP			5 (5.8%)
BWS			2 (2.3%)
Simpson-Golabi-Behmel Sd			1 (1.2%)
Duchene Myopathy			1 (1.2%)
PRETEXT stage	I	86	4 (4.6%)
	II		40 (46.5%)
	III		29 (33.7%)
	IV		13 (15.1%)
V-Hepatic vein and/or vena cava invasion	Yes	67	7 (10.4%)
	No		60 (89.6%)
P- Portal vein invasion	Yes	82	11 (13.4%)
	No		71 (86.6%)
E-Extra-hepatic invasion	Yes	77	4 (5.2%)
	No		73 (94.8%)
R-Ruptur at diagnosis	Yes	82	10 (12.2%)
	No		72 (87.8%)
F-multifocality	Yes	79	18 (22.8%)
	No		61 (77.2%)
Metastases at diagnosis		86	23 (26.7%)
AFP serum level at diagnosis (ng/ml)		86	442683 (11.5-2700000)
<100 ng/ml			1 (1.2%)
100-1000ng/ml			3 (3.5%)
CHIC risk stratification	Very-low/Low	86	38 (44.2%)
	Intermediate		10 (11.6%)
	High		38 (44.2%)
Chemotherapy	Cisplatin alone	80	33 (41.2%)
	Intensive polychemotherapy		47 (58.8%)
AFP serum level after neoadjuvant chemotherapy (ng/ml)		79	2799.5 (7-1111111)
Response to neo-adjuvant chemotherapy		79	62 (78.5%)
'Good-Responder'			
	Poor-Responder'		17 (21.5%)
Surgery	Partial liver resection	86	76 (88.4%)
	Liver transplantation		9 (10.5%)
	No surgery		1 (1.2%)
Status at the end of treatment: Complete remission		83	73 (88%)
	Residual disease		10 (12%)
Outcome during the FU	Progression	86	10 (11.6%)
	Relapse	86	18 (20.9%)
	Death	86	14 (16.3%)

Abbreviation: BWS=Beckwith Weidemann Syndrome; FAP= Familial adenomatous polyposis; Sd = syndrome;
Pretext= Pre-treatment extension disease; AFP= alpha-fetoprotein; FU = Follow up

diagnosis of HB and has been excluded of the disease specific survival analyses (considering only death due to HB progression). All 6 patients without neoadjuvant chemotherapy are in persistent complete remission. The 5-year time to progression (TTP) and disease-specific survival (DSS) were 69% (CI95: 59–79%) and 83% (CI95:76–92%), respectively (**Supp.** Fig. S2).

In univariate analysis, poor prognosis was associated with old age (≥ 3 years old at diagnosis, TTP: HR 4.1 (CI95: 1.9–8.8), p = 0.0003; DSS: HR 3.1 (CI95:1.1–8.8), p = 0.039), Pretext IV tumor (TTP: HR 2.7 (CI95: 1.2–6.2), p = 0.019; DDS: HR 3.5 (CI95: 1.2–10.5), p = 0.025), CHIC high-risk (TTP: HR 3.1 (CI95: 1.4–6.8), p = 0.0057; DSS: HR 3.4 (CI95:1.1–10.9), p = 0.038), poor response to neoadjuvant chemotherapy (TPP: HR 3.8 (CI95: 1.7–8.2), p = 0.0008; DSS: HR 8 (CI95: 2.6–24.7), p = 0.0003), and residual disease at the end of the initial treatment (after adjuvant chemotherapy, TTP: HR 15.1 (CI95: 6.1–37.7), p < 0.0001; DSS: HR51.1 (CI95:13–200), p < 0.0001) (Fig. 1). In contrast, metastases at diagnosis was associated with an increased risk of progression (HR: 2.7 (CI95:1.3–5.7), p = 0.0093) without significant increased risk of death (Fig. 1A). In the overall series of patients, neoadjuvant intensive polychemotherapy was associated with TTP and DSS compared with cisplatin alone but not with response to neoadjuvant chemotherapy (Fig. 1A). As CHIC stratification includes age, PRETEXT and AFP at diagnosis, we performed a multivariate analysis with the remaining feature evaluated at the surgery of the primary tumor, i.e. response to neoadjuvant chemotherapy. We showed that CHIC-High risk and poor response were independently related to survival (p = 0.0311 and p = 0.0016, respectively) (Fig. 1A).

2.2. *Cancer driver gene mutations and mutational signatures with clinical relevance*

The Wnt/β-catenin pathway was the most altered oncogenic pathway in HB (98.8%, n = 82/83, 3 tumors highly contaminated by normal cells were not analyzed). More precisely, *CTNNB1* was the most frequently mutated gene (91.6%, n = 76/83), followed by 6 *APC* mutations (including 5 germline) and *AXIN1* germline mutation in one case (**Supp.** Fig. S3). All *CTNNB1*, *APC* germline and *AXIN1* germline mutations were exclusive to each other (**Supp.** Fig. S3). Alterations in the Wnt/β-catenin pathway had no impact on prognosis (Fig. 2A). Alterations of the 11p15.5 locus were identified in 55/74 cases (74.3%) including chromosome 11p15.5 loss of heterozygosity (cnLOH, n = 31 or LOH, n = 2), IC1/IC2 methylation alterations (n = 19) or *CDKN1C* alterations (n = 3), and 11p15.5 locus alteration was associated to a higher risk of progressive disease (p = 0.040) (Fig. 2A). Mosaic 11p15.5 locus alterations were also identified in the non-tumor liver tissues of 12/70 cases (17%), without impact on prognosis (TTP log-Rank p = 0.74; DSS log-Rank p = 0.89) according to Pilet et al[20]. Beyond the Wnt/β-catenin pathway and 11p15.5 locus alterations, *TERT* promoter mutation (n = 9/81) was more frequently identified in old children (p < 0.0001) and in tumors with macrotrabecular architecture (p = 0.0009), without association with response to neoadjuvant chemotherapy and survival (**Supp.** Fig. S3-S4).

Regarding rare driver mutations in univariate analysis using the COX model, *NFE2L2* mutation (n = 4/83) and *NF1* mutation (n = 3/63) were associated with a poor TTP (p = 0.0003 and p < 0.0001) and DSS (p < 0.0001 and p = 0.043), and a poor-response to neoadjuvant chemotherapy according to the clinical definition (p = 0.0019 and p = 0.023) (Fig. 2A-B). In contrast, *MDM4* amplification (n = 5/64) was associated with more frequent HB progression (p = 0.0003) but not with survival (p = 0.96) and response to neoadjuvant chemotherapy (p = 0.21) (Fig. 2A). Nevertheless, all these results concerned few patients (**Supp.** Fig. S3). In order to be more relevant in our analyses, we considered all the rare driver mutations together. A total of 24 tumors out of 65 demonstrated at least one mutation in either *NFE2L2, RPS6KA3, GPC3, NF1, CREBBP, BCORL1, ASXL1, DDX3X, LEF1, ARID1A, NOTCH1*, *MDM4, FGF19/CCND1*, *KEAP1*, or *IRF2* (**Supp.** Fig. S3); interestingly these children showed poor TTP (p = 0.0005) and DSS (p = 0.0092), and were enriched in 'Poor-Responders' to neoadjuvant chemotherapy (p = 0.0017) (Fig. 2A-B). The presence of a rare driver mutation was not associated with the age at diagnosis (p = 0.70).

SBS35 cisplatin mutational signature was identified in 33.3% of the tumors resected after neoadjuvant chemotherapy (n = 18/54) and associated with TTP (p = 0.015) and DSS (p = 0.027) (Fig. 2A-B). As expected, SBS35 cisplatin mutational signature was not identified in the 7 samples from tumor without neoadjuvant chemotherapy (6 patients resected at diagnosis and 1 patient with only a biopsy at diagnosis who died before surgery). Regarding the response to neoadjuvant chemotherapy, 56% of patients with an SBS35 cisplatin mutational signature were 'Poor-Responders' while only 11% of patients without SBS35 were 'Poor-Responders' (10/18 vs 4/35, p = 0.001) (Fig. 2B).

A

		TTP univariate			DSS univariate			DSS Multivariate		
		HR	CI95	p-value	HR	CI95	p-value	HR	CI95	p-value
Age at diagnosis ≥3 years	28/86	4.11	(1.9-8.8)	0.00027	3.1	(1.1-8.8)	0.039			
Gender: female	30/86	1.3	(0.6-2.8)	0.49	2.0	(0.7-5.8)	0.19			
PRETEXT IV	13/86	2.7	(1.2-6.2)	0.019	3.5	(1.2-10.5)	0.025			
Multifocality	18/79	3.0	(1.4-6.7)	0.061	2.7	(0.8-8.5)	0.092			
Metastases at diagnosis	23/86	2.7	(1.3-5.7)	0.0093	2.1	(0.7- 6.0)	0.17			
High-risk CHIC stratification	38/86	3.1	(1.4-6.8)	0.0057	3.4	(1.1-10.9)	0.038	3.7	(1.1-12.1)	0.031
Neoadjuvant polychemotherapy	47/80	2.4	(1-5.6)	0.050	4.7	(1-21)	0.043			
'Poor-Responder' to neoadjuvant chemotherapy	17/79	3.8	(1.7-8.2)	0.00078	8.0	(2.6-24.7)	0.00028	8.8	(2.8-27.5)	0.00016
No complete remission at the end of initial treatment	10/83	15.1	(6.1-37.7)	<0.0001	51.1	(13-200)	<0.0001			

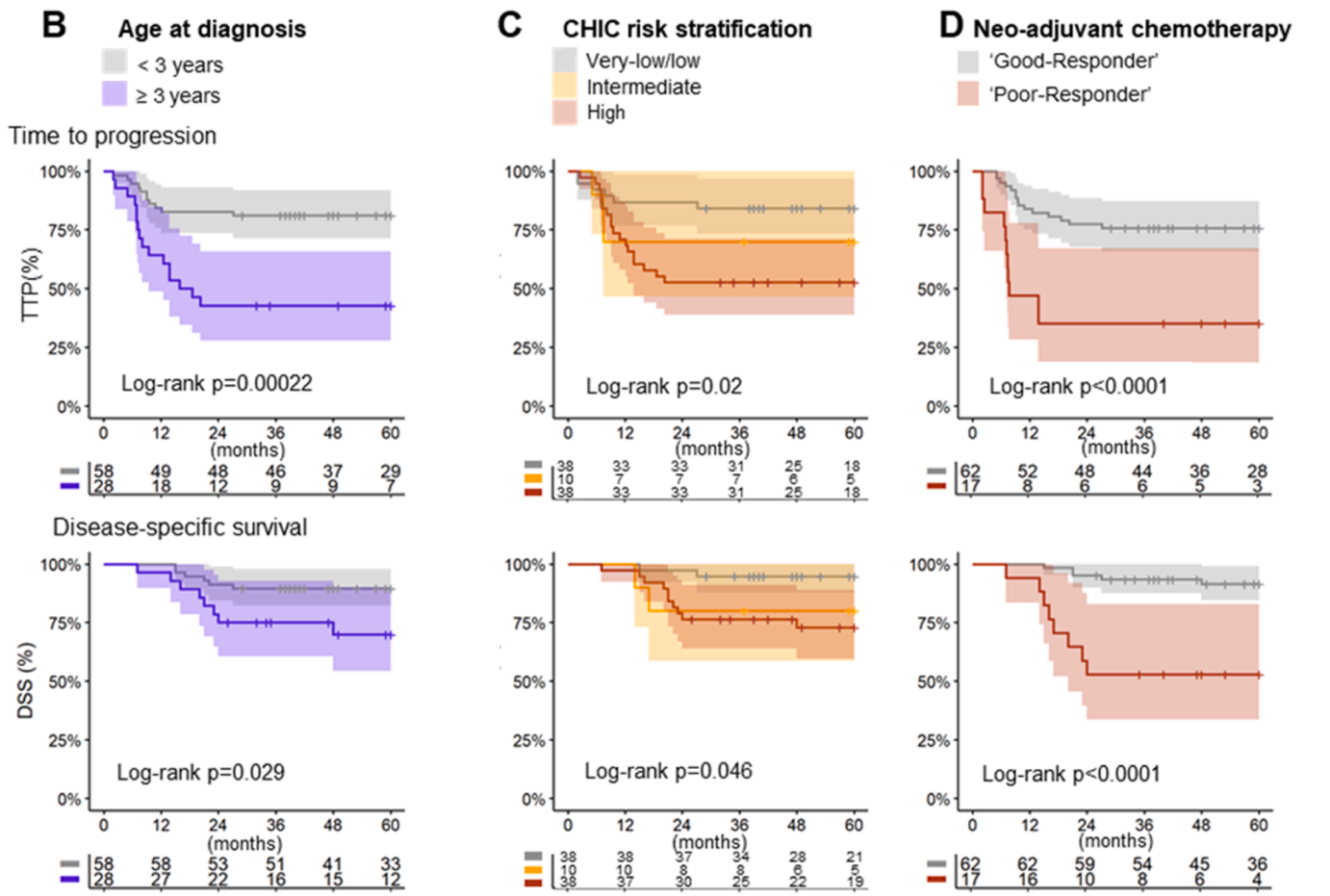

Fig. 1. Survival analysis of clinical data of hepatoblastoma cohort. A) Univariate and multivariate[a] analysis of clinical data for time to progression (TTP) and disease-specific survival (DSS) in 86 hepatoblastomas using Cox Model. B-D) Time to progression (TPP) and disease specific survival (DSS) according to clinical data (n = 86) using log-rank test and Kaplan-Meier: B) age at diagnosis, C) CHIC risk stratification, and **D**) response to neo-adjuvant chemotherapy. [a]Due to the low number of events in our series (death=14), to evaluate the independent predictive value of the DSS risk factors, bivariate analysis has been realized between the most representative clinical variable and the poor response to neoadjuvant chemotherapy.

2.3. Histo-molecular phenotype of HB with clinical relevance

We classified 85/86 HB tumors of our series according to our 3-subtypes transcriptomic classification and immune classification as already described in Hirsch et al [17]. We identified tumors belonging to our transcriptomic classification ('Hepatocytic', 'Liver Progenitor', 'Mesenchymal') subtypes with a close correlation with Cairo classifications (C1/C2), Hooks classifications (C1/C2A/C2B), and Nagae classifications (Hepatocyte, Proliferative, Mesenchymal) (all $p < 0.0001$) (**Supp.** Fig. S5). Transcriptomic classification was also closely associated with the majority of histological component of HB in 85 patients' samples ($p < 0.0001$): 15/16 embryonal samples were classified 'Liver

A

		TTP			DSS		
		HR	CI95	p-value	HR	CI95	p-value
CTNNB1	76/83	0.6	(0.2-2.1)	0.47	>10^7	(0-inf)	1
*APC**	6/67	0.8	(0.2-3.4)	0.76	0	(0-inf)	1
11p15.5 locus alteration**	55/74	4.6	(1.1-19.7)	0.040	4.2	(0.5-32.7)	0.17
TERT promoter	9/81	1.9	(0.7-5.2)	0.19	4.5	(0.3-6.6)	0.62
Rare drivers mutation***	24/65	4.4	(1.9-10.1)	0.0005	4.8	(1.5-15.6)	0.0092
NFE2L2	4/83	8	(2.6-24.6)	0.0003	14.6	(3.8-56.1)	<0.0001
MDM4	5/64	6.7	(2.4-19.1)	0.0003	1.1	(0.1-8.1)	0.96
GPC3	3/63	2.5	(0.6-10.8)	0.21	4.4	(0.9-19.8)	0.056
*NF1****	3/63	24	(5.2-109.7)	<0.0001	4.8	(1.1-22.4)	0.043
SBS35 cisplatin mutational signature (after neoadjuvant chemotherapy)	18/54	2.9	(1.2-6.9)	0.015	3.7	(1.2-11.7)	0.027

*including 5 patients with germline mutation
**including 12 mosaic alteration in normal liver
****NFE2L2*, *RPS6KA3*, *GPC3*, *NF1*, *CREBBP*, *BCORL1*, *ASXL1*, *DDX3X*, *LEF1*, *ARID1A*, *NOTCH1 KEAP1*, *MDM4*, *FGF19/CCND1*, or *IRF2*
****including 1 patient with germline mutation

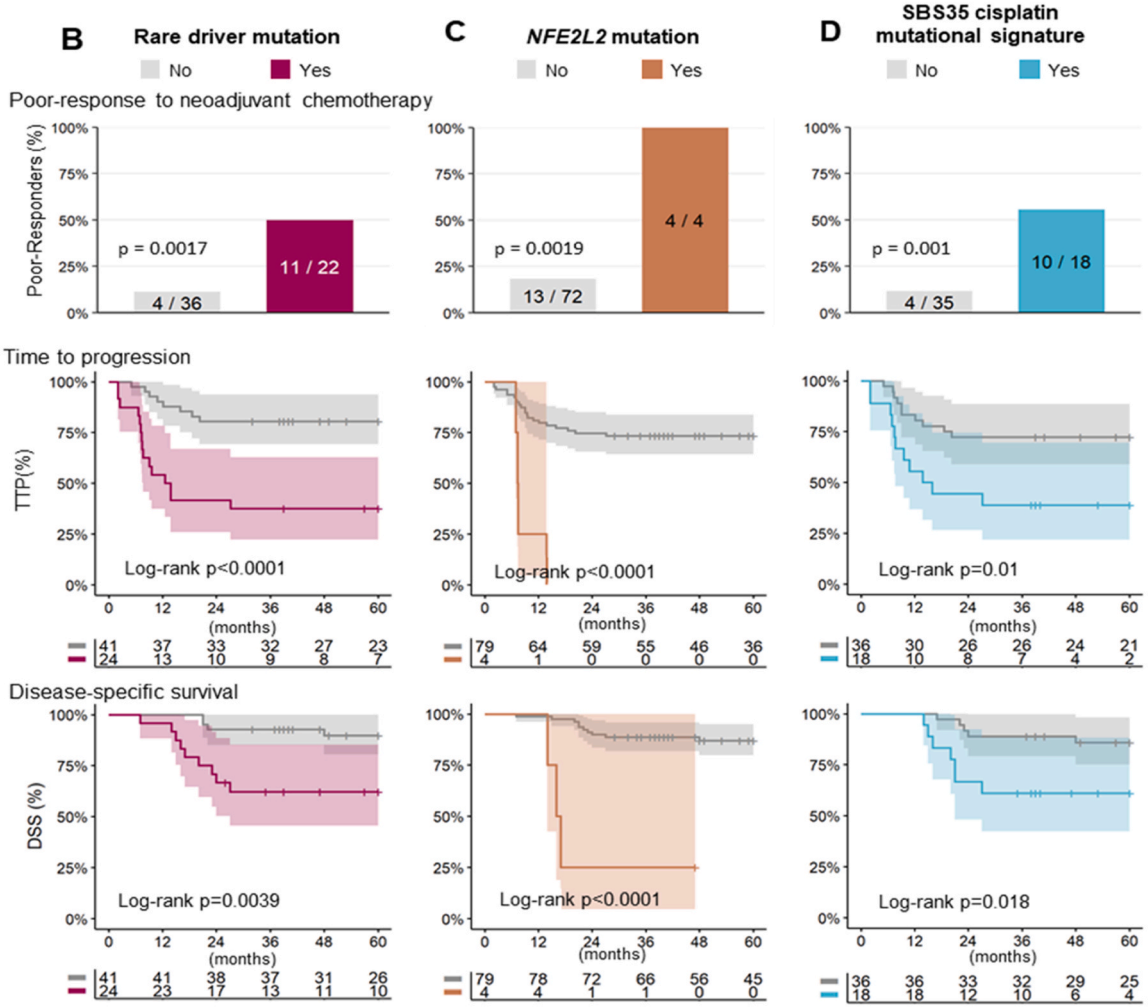

Fig. 2. Survival analysis of mutational data of hepatoblastoma cohort. A) Univariate analysis of gene mutations for time to progression (TTP) and disease-specific survival (DSS) in 86 hepatoblastomas using Cox Model. B-C) Response to neoadjuvant chemotherapy, time to progression (TTP) and disease-specific survival (DSS) according to mutational data using log-rank test and Kaplan-Meier: B) presence of rare driver mutations, C) *NFE2L2* mutation, and D) SBS35 cisplatin mutational signature after neoadjuvant chemotherapy.

Progenitor', 43/58 fetal samples were classified hepatocytic and 11/11 mesenchymal samples were classified mesenchymal (**Supp.** Fig. S5).

In the survival analysis, patients with 'Liver Progenitor' and 'Immune Cold' tumors after neoadjuvant chemotherapy had a poorer TTP (log-rank p = 0.00045 and log-rank p = 0.0015, respectively) and DSS (log-rank p = 0.00049 and log-rank p = 0.0011, respectively). They were mostly 'Poor-Responders' to neoadjuvant chemotherapy (12/24 LP vs 4/53 non-LP, and 14/30 cold vs 2/48 hot, $p < 0.0001$) and frequently harbored SBS35 cisplatin mutational signature (17/22 LP vs 1/32 non-LP, and 18/26 cold vs 0/28 hot, $p < 0.0001$) (Fig. 3A-B). Since HB tumors are highly heterogeneous, we tested if the histological reviewing of the whole surgical specimen after neoadjuvant chemotherapy could reflect the 'Liver Progenitor' molecular subtype and predict survival [17]. Tumors with an embryonal predominant type at histology

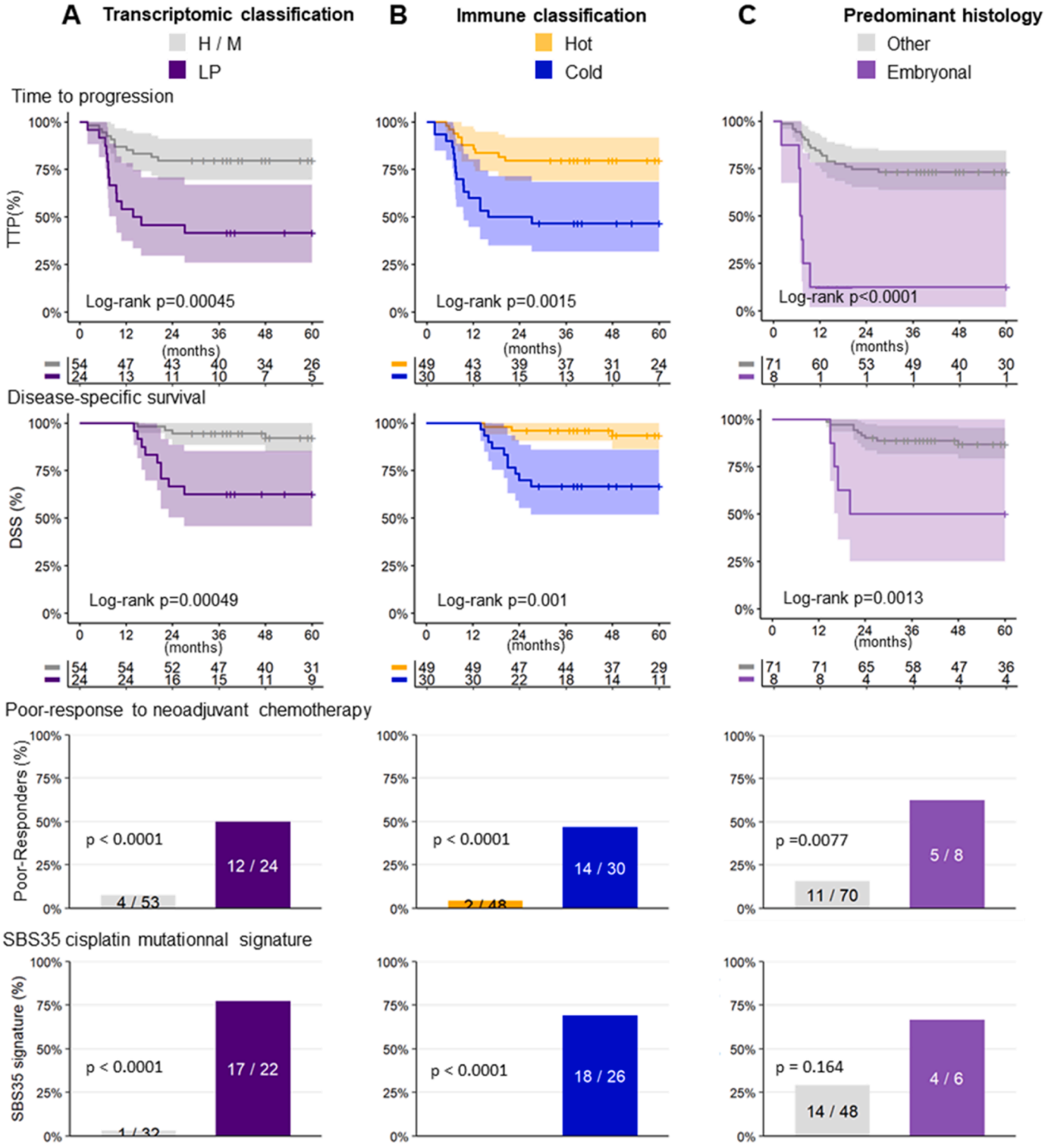

Fig. 3. Survival analysis of histo-molecular data of hepatoblastoma cohort. A-C) Time to progression (TTP), disease-specific survival (DSS) and response to neo-adjuvant chemotherapy according with the histo-molecular phenotype for patients resected after neoadjuvant chemotherapy (n = 79): 'Liver Progenitor' subtype (A), immune score (B), embryonal predominant type at histology on surgical specimen after neoadjuvant chemotherapy (C).

(n = 8/79, 10.1%) had a poorer TTP (log-rank p < 0.0001) and DSS (log-rank p = 0.0013) (Fig. 3C), and were enriched in 'Poor-Responders' patients to neoadjuvant chemotherapy (p = 0.0077). Of note, among the 6 HB resected before chemotherapy, only one of them showed an embryonal predominant type at histology, and all survived without progressive disease (**Supp.** Fig. S5).

2.4. Absolute AFP serum value after neoadjuvant chemotherapy is associated with chemoresistance and histo-molecular features

Absolute AFP level at the end of chemotherapy is influenced by many factors, including initial level and response to chemotherapy. We hypothesized that this absolute level could also reflect some biological features of HB. Therefore, we analyzed HB features ranked according to

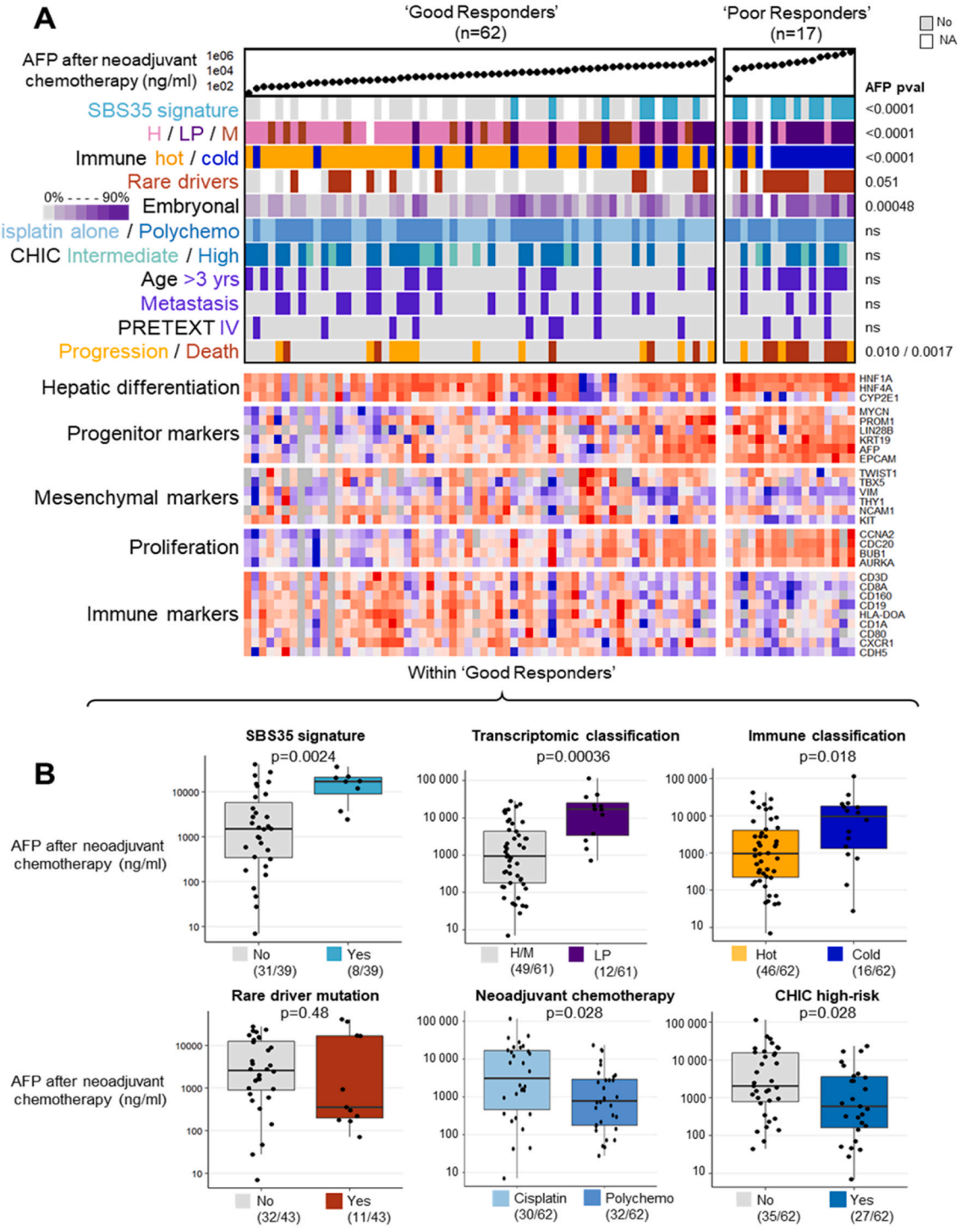

Fig. 4. Clinical, mutational, and histo-molecular features according with response to neoadjuvant chemotherapy. A) Heatmap representation of absolute AFP after neoadjuvant chemotherapy and good/poor response to neoadjuvant chemotherapy, and univariate analysis of absolute AFP after neoadjuvant chemotherapy among the features. The heatmap also shows mRNA expression in 77 HB for 28 key genes representative of hepatic differentiation, progenitor, mesenchymal and proliferation markers, as well as markers of immune infiltration. B) Association of absolute AFP after neoadjuvant chemotherapy among the features within the 'Good-Responder' patients.

the medical definition of good and poor responses (see material and methods), and absolute AFP serum value after neoadjuvant chemotherapy (Fig. 4). As expected, AFP at the end of neoadjuvant chemotherapy was higher in 'Poor Responders' patients compared to 'Good Responders' patients ($p < 0.0001$). Tumors meeting one of the 3 molecular features associated with chemo-resistance, 'SBS35 mutational signature', 'Liver Progenitor' transcriptomic subtype, or 'Immune Cold' were associated with higher absolute AFP serum value at the end of neoadjuvant chemotherapy in the overall cohort of patients ($p < 0.0001$, Fig. 4 A) and in the subgroup of 'Good Responder' patients (Fig. 4B). Among patients with CHIC high-risk, 27/35 obtained a good response after neoadjuvant chemotherapy, they were associated with a lower AFP at the end of neoadjuvant chemotherapy ($p = 0.028$), an 'Immune Hot' ($p = 0.00042$) and 'Hepatocytic' transcriptomic phenotype ($p = 0.018$) compared to the CHIC high-risk 'Poor-Responders' patients.

2.5. Integration of clinical and molecular features associated with prognosis and response to chemotherapy

To integrate associations with prognosis and response to neoadjuvant chemotherapy, we identified two major clusters of features in a correlogram according to Fisher test (Fig. 5). Cluster 1 included closely related features (older age, presence of metastases, PRETEXT IV, High risk CHIC stratification, and *TERT* promoter mutation), it was associated with poor survival but not with response to neoadjuvant chemotherapy (Fig. 5). In contrast, cluster 2 included 'Liver Progenitor', 'Immune Cold' subtypes, mutations in rare drivers, SBS35 cisplatin mutational signature, and embryonal predominant type at histology, these features were highly associated together, but also with both poor survival and poor response to neoadjuvant chemotherapy (Fig. 5). Interestingly, in multivariate analysis, molecular phenotypes after neoadjuvant chemotherapy were associated with DSS independently of clinical features ($p < 0.05$) (Fig. 5 and **Supp** Fig. S6). In addition, we showed that presence of rare driver mutation was associated with DSS independently of other molecular features ($p < 0.05$) (**Supp** Fig. S6).

3. Discussion

In this study, we highlighted new molecular features (rare driver mutations, SBS35 mutational signature of cisplatin resistance, 'Liver Progenitor' and 'Immune Cold' transcriptomic subtypes) of post-chemotherapy HBs predictive of chemoresistance, progression, and death independently of the known clinical features of aggressiveness at diagnosis such as age, PRETEXT, or CHIC stratification. A better understanding of the molecular bases associated with poor survival and poor response to neoadjuvant chemotherapy could help to identify earlier patients resistant to chemotherapy and adapt adjuvant therapy decisions. In the spirit of translating the molecular biomarkers into clinical practice, we showed that a major component of embryonal histology in the pathological reviewing of the tumor and the slow decrease of AFP during neoadjuvant chemotherapy are reliable markers to predict chemoresistance and survival, that remain to be validated in prospective series of treated patients. Our perspectives for translation in clinical practice are 1) quantification of the embryonal / Liver Progenitor / Immune cold sector, 2) readout of resistance to cisplatin using SBS35 assessment in clinical practice to perform a temporal follow-up during treatment, either in tumor or liquid biopsies, and 3) interest of

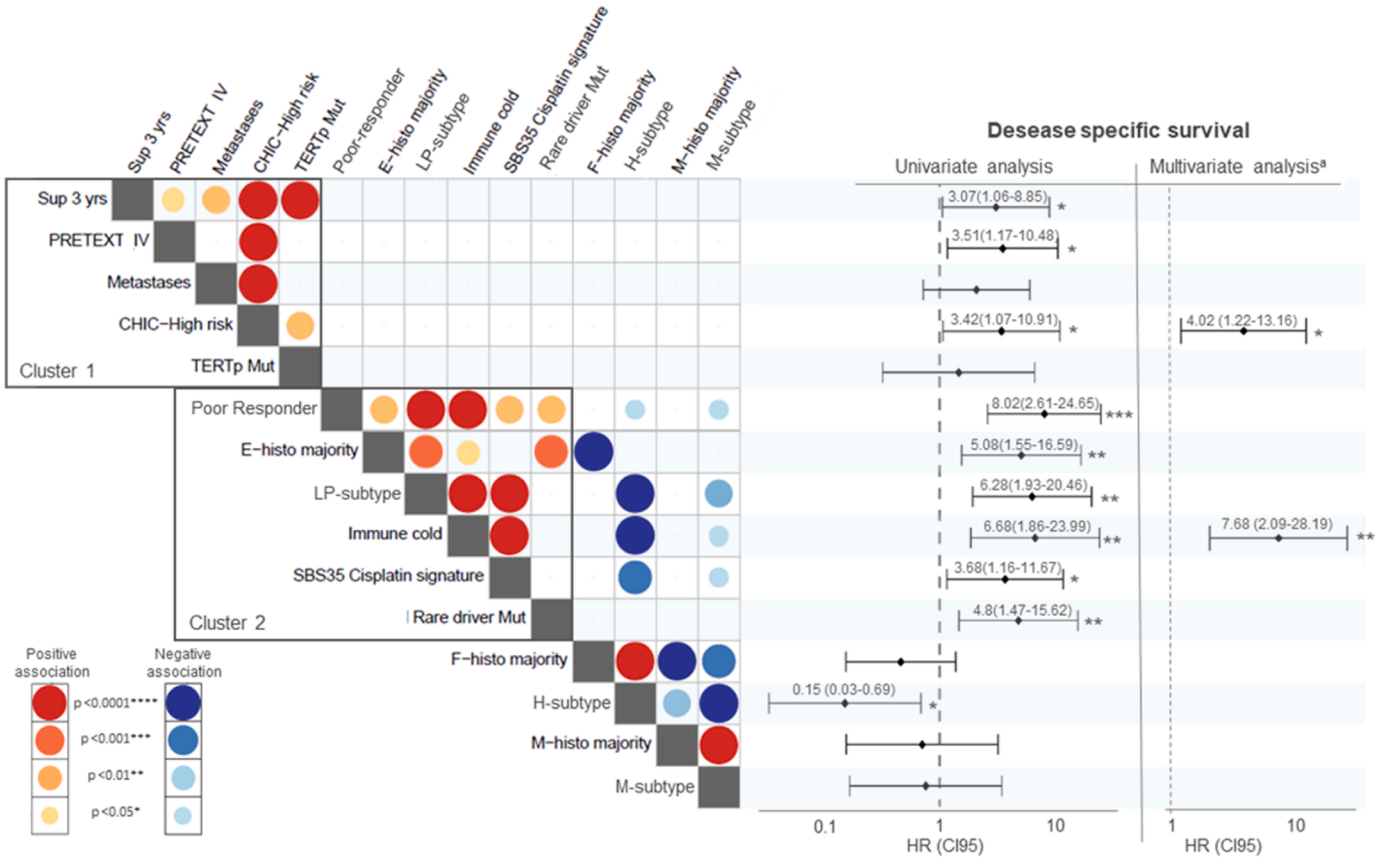

Fig. 5. Integration of clinical, mutational, and histo-molecular analysis. Correlogram of Fisher test, univariate, and multivariate[a] analysis using Cox model of disease-specific survival of clinical, histo-molecular, and mutational features in hepatoblastoma series (n = 86). [a]Due to the low number of events in our series (death=14), to evaluate the independent predictive value of the DSS risk factors, bivariate analysis has been realized between the most representative clinical variable and the poor response to neoadjuvant chemotherapy.

using novel therapeutic approaches directly targeting the chemo-resistant Liver Progenitor cells, for instance with inhibitors of PLK1, CHEK1 or Survivin as described in Hirsch et al, Cancer Discov. 2021.

Currently, a major objective to improve the multidisciplinary care of children with HB is to identify more accurately patients the most at risk of recurrence, chemoresistance, and poor prognosis[2–8]. In the present study, our cohort of 86 patients was representative of classical HB patients treated in western countries, with a similar sex-ratio of 2:1 but slightly enriched in older patients and metastatic disease at diagnosis [5]. All patients included were diagnosed as hepatoblastoma by expert pathologists, independently of age, but we cannot exclude that potential HCN-NOS (Hepatocellular Neoplasm Not Otherwise Specified) are included in the analysis since this diagnosis is debated among pathologists. In our cohort, CHIC high-risk patients, according to the most recent system of risk stratification, were associated with progression and survival but not with resistance to the initial neoadjuvant chemotherapy [3,11,13]. According to the SIOPEL recommendations, most of the children in our series (93%) were treated with neoadjuvant cisplatin-based chemotherapy followed by surgery and adjuvant chemotherapy [2,5,12,30–33]. This historically therapeutic strategy is different from the US where some of the children are treated first by surgery [4,34,35]. In this context, our cohort of patients enabled to collect many tumor samples after neoadjuvant chemotherapy and thus to deeply investigate the initial mechanisms of resistance to treatment [2,30,33,36,37].

While ß-catenin signaling pathway was activated by a mutation of either *CTNNB1*, *APC* or *AXIN1* in all tumors except one, *CTNNB1* mutations were not associated with prognosis as in other cohorts of patients [11,26,27,38]. In contrast, even if 11p15.5 alterations are also frequently detected in HB, they were associated with an increased risk of progression. Interestingly, *TERT* promoter alterations that are found in 11% of HB, mostly in older patients, were not associated with prognosis, this result contrasts with previous studies[10,21,38]. However, *TERT* promoter mutations were closely related to children age but not to the prognosis. Beyond these common alterations, rare driver gene mutation ($< 10\%$) were gathered together, and were found in 37% of HB patients, associated with chemoresistance and poor survival. Among these rare driver genes altered in HB, *NFE2L2* gene mutation (5%) appeared to be the most closely associated with poor survival [10,11,16,39]. Altogether, larger cohorts of HB patients should be monitored in order to evaluate the prognosis impact of each rare driver gene in pre- and post-chemotherapy. A key question is to understand if rare driver mutations seen in post-chemotherapy HB were already present at the time of diagnosis, or if they were selected/induced by resistance to cisplatin. Of note, in the Japanese cohort of 103 HB analyzed before chemotherapy [19], only 17% of tumors harbored a rare driver alteration, as opposed to 37% in the French cohort of post-chemotherapy tumors enriched in HB resistant to chemotherapy. This result could be influenced by (1) enrichment of HB resistant to chemotherapy in the French analyzed cohort, and (2) the selection/induction of mutations in tumors resistant to chemotherapy after neoadjuvant treatment (**Supp** Fig. S7). Furthermore, in the Japanese cohort, rare driver alterations were not associated with progression and survival, highlighting the importance of analyzing post-chemotherapy samples for predictive molecular features (**Supp** Fig. S7). These results should be validated in a matched pre- vs post-chemotherapy enlarged cohort. In the present study, we identified additional molecular markers related to tumor response and to survival. Among them, two transcriptomic subtypes 'Liver Progenitor' and 'Immune Cold' were associated with poor survival and resistance to neoadjuvant chemotherapy. Also, the SBS35 mutational signature, that we previously identified as a hallmark of HB tumor cells proliferating under chemotherapy, was mainly identified in tumor of 'Liver Progenitor' and 'Immune Cold' subtypes[17]. The mechanism of SBS35 mutation accumulation is due to the ability of the 'Liver Progenitor' tumor cells to bypass cisplatin-induced DNA adducts due to the overexpression of various DNA polymerases and DNA repair factors involved in double strands DNA repair[17,40–42]. Here we showed that SBS35 was not only related to survival but also to non-response to neoadjuvant chemotherapy suggesting that SBS35 detection could be an early marker of the risk of resistance to chemotherapy.

Evaluation of the risk of progression, relapse, and death is crucial to define the best therapeutic scheme, with intensification or de-escalading adjuvant chemotherapy treatments adapted to patient and tumor profiles. In the objective to introduce molecular markers into the clinics, translation to surrogate histological and biological features could be a useful strategy. In this line, we propose that the histological 'embryonal' pattern could be a reliable marker of the 'Liver Progenitor' tumors. However, since histological and molecular heterogeneity in HB is huge, quantification of the 'embryonal' tumor areas could be important on the surgical specimen after chemotherapy [1,13,14,43]. It could be evaluated to adapt adjuvant chemotherapy after surgery of the primary tumor, or at diagnosis as proposed by Zhou and collaborators in CHIC high-risk patients with pure embryonal pattern at biopsy [13]. A similar strategy is already implemented for nephroblastoma, in which predominance of blastemal cancer stem cells is classified as an independent category of poor prognosis [44,45]. Our study included 86 patients with available clinical data and molecular data from sample after neoadjuvant chemotherapy. It is significant for a very rare disease like HB, however, it is also a limitation and the proposed features associated with resistance to chemotherapy and survival remain to be validated in large prospective cohorts of worldwide patients.

In conclusion, response to neoadjuvant chemotherapy and survival are associated to genomic and pathological features independently of the known clinical features. Their introduction in clinical care should be discussed in the future after a validation of these results in a larger prospective series of patients.

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editing. **Taque Sophie:** Resources, Validation, Writing – review & editing. **Hirsch Theo:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Resources, Software, Supervision, Validation, Writing – original draft, Writing – review & editing. **Fresneau Brice:** Data curation, Methodology, Resources, Validation, Writing – review & editing. **Morcrette Guillaume:** Data curation, Validation, Writing – review & editing, Resources. **Pilet Jill:** Data curation, Formal analysis, Validation, Writing – review & editing. **Cornet Marianna:** Data curation, Validation, Writing – review & editing. **Rebouissou Sandra:** Data curation, Methodology, Validation, Writing – original draft. **Sarnacki Sabine:** Resources, Validation, Writing – review & editing. **Faure-Conter Cécile:** Resources, Validation, Writing – review & editing. **Aerts Isabelle:** Resources, Validation, Writing – review & editing. **Pire Aurore:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Chardot christophe:** Resources, Validation, Writing – review & editing. **Capito Carmen:** Resources, Validation, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ejca.2024.113583.

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