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# Role of Allelic Imbalance in Predicting Hepatocellular Carcinoma (HCC) Recurrence Risk After Liver Transplant

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**Background:** One of the most controversial problems for liver transplantation in patients affected by hepatocellular carcinoma (HCC) remains the lack of an oncologic staging system to predict cancer recurrence after liver transplantation (LT). We analyzed allelic imbalance (AI) in 19 microsatellites, and assessed the post-LT HCC recurrence risk.

**Material/Methods:** Seventy-one patients were included; 18 had tumor recurrence within 5 years post-transplant. Molecular analysis was done in the primary HCC and peripheral blood samples: a total of 19 microsatellites was used to assess AI. Specific AI was evaluated when outside of range value between 0.66 and 1.5. Based on data in the literature, we grouped the 19 microsatellites into 4 panels. We calculated the fractional allelic imbalance (FAI) to make comparisons between different panels including different subsets of microsatellites.


**Results:** We report that AI was associated with HCC recurrence in 3 main *loci* (D3S2303, D9S251, and D9S254). Tumor recurrence was associated only with 2 specific panels with 9 microsatellites previously reported to be associated with high risk for HCC recurrence. Our data show that fractional allelic imbalance (FAI) index has good negative ability to predict HCC recurrence (Panel 2: negative predictive value of 95%).

**Conclusions:** AI analysis could have prognostic value in risk management of HCC recurrence after LT, especially for early recurrence.

**MeSH Keywords:** Allelic Imbalance • Carcinoma, Hepatocellular • Liver Transplantation • Treatment Outcome

**Abbreviations:** AI – allelic imbalance; AR – allelic ratio; AUC – area under the ROC curve; CT – computerized tomography; FAI – fractional allelic imbalance; FFPE – formalin-fixed paraffin-embedded; HCC – hepatocellular carcinoma; LOH – loss of heterozygosity; LT – liver transplantation; MELD – model for end-stage liver disease; MRI – magnetic resonance imaging; NPV – negative predictive value; OR – odds ratio; PPV – positive predictive value; RFS – recurrence-free survival; ROC – receiver operative characteristic; TNM – tumor-node-metastasis

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## Background

Over the last 20 years hepatocellular carcinoma (HCC) has become one of the most common indications for liver transplantation (LT). Data from the European Liver Transplant Registry from 1988 to 2015 show that cancer represents 16.5% of overall causes of LT, and 87.3% are HCC-related. Survival analysis demonstrates that long-term survival (5 or more years after LT) of this specific subset of patients is lower than that of recipients with other diseases [1], as also reported by the Organ Procurement and Transplantation Network in the USA [2].

In a seminal paper published in 1997, Mazzaferro et al. established what came to be called the Milan criteria, an attempt to standardize criteria for transplantation of patients with HCC. They reported that patients with either 1 tumor with a diameter  $\leq 5$  cm, or patients with 2/3 tumors each with a diameter  $\leq 3$  cm, had a recurrence-free survival (RFS) rate at 5 years of 75% and 83%, respectively [3]. The Milan criteria have been validated by several groups, and were adopted by the United Network for Organ Sharing UNOS staging system for allocating organs, although the criteria have also been criticized for being too restrictive and for limiting the prognostic information to only radiologic appearance, without considering other clinical, molecular, or pathologic data [4]. Other research groups have tried to extend the Milan criteria, some based on dimensional features [5–8], while others have considered microvascular invasion [9,10] or tumor grade [11], or evaluating biochemical parameters [12–14]. Mazzaferro et al. tried to simplify data, first with the Metroticket Paradigm [15], and then by proposing the Up-to-7 Criteria [16].

Nonetheless, the debate over the criteria for transplantation of HCC patients is still far from over. Some recent studies have defined loss of heterozygosity (LOH) or allelic imbalance (AI) according to the level of gene damage and genomic instability, and explored whether a panel of tumor-suppressor gene markers could be useful in predicting recurrence with an index of cumulative mutational damage (fractional allelic imbalance, FAI) [17–19].

Other studies have reported that panels including specific microsatellites were significantly correlated with the post-transplant recurrence-free survival (RFS), but none achieved perfect discrimination alone [20,21], and proposed a rational approach using FAI in conjunction with models based on clinical data to expand the conventional LT selection policies [22].

Recently, Schmidt, and Marsh, at UPMC, proposed integrating data from FAI and macrovascular invasion, developing a novel staging system predicting HCC recurrence after LT (Pittsburgh staging system) [23].

Our study aimed to analyze the microsatellite LOHs to assess the risk of tumor recurrence after LT in recipients with HCC, in order to help move the field away from morphometric criteria to ones that are based on molecular information on tumor biology.

## Material and Methods

### Patients

The study population included 71 orthotopic LT recipients affected by HCC at IRCCS – ISMETT, in Palermo, Italy between 2006 and 2012. Severity of chronic liver disease was based on the Model for End-stage Liver Disease score (MELD). Histopathological findings were used to determine the tumor stage, classified by the tumor-node-metastasis (TNM) staging system. To monitor hepatic recurrence or distant metastasis, all patients were routinely followed up for at least 3 years with blood tests, magnetic resonance imaging (MRI), and/or computerized tomography (CT) scans. ISMETT's Institutional Research Review Board and local Ethics Committee approved the research protocol (approval number IRRB/26/11, signed by Mariolina Crisci, 5<sup>th</sup> December 2016).

### Microsatellite analysis

Genomic DNA was extracted from whole blood, formalin-fixed paraffin-embedded (FFPE), amplified using Type-it Microsatellite PCR kit (QIAGEN), and separated by capillary electrophoresis on the 3500 Genetic Analyzer (ThermoFisher). A total of 19 microsatellites were used to assess AI, which included 9 *loci* previously reported as significantly associated with a high tumor recurrence risk after LT [18, 24], 6 *loci* that may be associated with recurrence but were excluded from the UPMC group [23], and an additional 4 *loci* suggested as indicative of recurrence [23].

### Identification of microsatellites panels

Based on data in the literature, we grouped the analyzed microsatellites into 4 different panels (Table 1) to assess the association between FAI and the presence of tumor recurrence. Panel 1 (9 *loci*) included the 9 microsatellites previously reported as associated with a high risk for HCC recurrence [24]. Panel 2 (15 *loci*) included an additional 6 microsatellites that may be associated with HCC recurrence [20]. Panel 3 (13 *loci*) included, in addition to the previous 9 *loci*, another 4 microsatellites suggested to be indicative of HCC recurrence (personal communication by Dr. Marsh, from UPMC). Panel 4 (19 *loci*) included all selected microsatellites (Table 1).

**Table 1.** Microsatellites and panels.

Gene	Locus	Microsatellite	Panel 1	Panel 2	Panel 3	Panel 4
L-myc	1p33	D1S162			x	x
L-myc	1p34	MYCL5NT	x	x	x	x
L-myc	1p35	D1S1161			x	x
CMM	1p36	D1S407	x	x	x	x
OGG1	3p24	D3S2303		x		x
VHL	3p26	D3S1539	x	x	x	x
APC	5q21	D5S615	x	x	x	x
MCC	5p21	D5S592		x		x
PTCH	9q22	D9S252		x		x
CDKN2A/p16	9p21	D9S251	x	x	x	x
CDKN2A/p16	9p21	D9S254		x		x
PTEN	10q23	D10S520		x		x
PTEN	10q23	D101173		x		x
TP53	17p13	D17S1289	x	x	x	x
TP53	17p13	D17S974	x	x	x	x
TP53	17p13	TP53 L1	x	x	x	x
TP53	17p23	D17S786			x	x
TP53	17p23	D17S516			x	x
DCC/SMAD4	18q21	D18S814	x	x	x	x

### Allelic imbalance analysis

An AI analysis investigated and compared healthy tissue (peripheral blood samples) and diseased tissue in the primary HCC. Microsatellite markers were designated as informative/heterozygous (2 different alleles/peaks) or non-informative/homozygous (1 allele/peak excluded for frequency calculation of AI). For any informative microsatellite, signal intensity in tumor DNA was compared with those of the corresponding normal DNA; the allele ratio (AR) between 2 allele peaks for each marker for each sample (peak height of allele 1/peak height of allele 2) was calculated, and AI was subsequently obtained as the ratio between the AR of the healthy sample and the AR of the diseased sample. Alleles were considered to be in balance when AI values were between 0.66 and 1.50 [21]. Two different grades of LOH were considered: 1) presence of LOH when AI values were either 0.50–0.65 or 1.51–2.00; 2) high-level LOH when AI values were either below 0.50 or greater than 2.00, respectively [18–29].

To make comparisons between different panels including different subsets of microsatellites, we calculated the FAI as the proportion of informative (heterozygous) microsatellites that exhibit LOH over the total number of informative *loci*.

### Statistical analysis

Continuous variables are expressed as mean and inter-quartile range (IQR), while categorical variables are expressed as frequency and percentage. Differences in the rates of HCC recurrence were tested by Fisher's exact test or Pearson's chi-square test without Yates correction, as appropriate; odds ratio (OR) was also used to assess the relationships. Predictive performances of different microsatellite panels were evaluated in terms of negative predictive value (NPV), positive predictive value (PPV), sensitivity, and specificity; the best discriminating cut-off value (Youden's index) was established for the FAI. GraphPad Prism 6, MedCal, and EpiTools were used for all statistical analyses.

## Results

### Clinical data and HCC recurrence

Of the 71 patients, 59 (83%) were male, and HCV infection was the main cause of liver disease. TNM classification of HCC was available in 52 patients. Forty-eight patients had multiple tumors involving 1 or both liver lobes, and 29 had

**Table 2.** Clinical and pathological data: Association between clinical data and HCC recurrence. Fisher's exact test and Pearson's chi-square (chi-square without Yates correction) were used, as appropriate.

Clinical and pathological data	Overall	No HCC Recurrence	HCC Recurrence	p-Value
n	71	53	18	
Male Gender, no. (%)	59 (83.1)	44 (83.0)	15 (83.3)	1.000
Age, median [IQR]	59.00 [53.5, 63.0]	59.0 [52.0, 63.0]	59.0 [56.0, 61.8]	0.706
HCC etiology, no (%)				0.216
HBV	14 (19.7)	13 (24.5)	1 (5.6)	
HBV+HCV	1 (1.4)	1 (1.9)	0 (0.0)	
HCV	49 (69.0)	35 (66.0)	14 (77.8)	
Other	7 (9.9)	4 (7.5)	3 (16.7)	
MELD, median [IQR]	12.00 [9.0, 15.0]	12.00 [9.0, 15.0]	11.5 [9.0, 14.8]	0.801
HCC nodules, no. (%)				0.128
1	23 (32.4)	19 (35.8)	4 (22.2)	
2	19 (26.8)	16 (30.2)	3 (16.7)	
3+	29 (40.8)	18 (34.0)	11 (61.1)	
Largest nodule size (cm), median [IQR]	2.50 [2.0, 3.5]	2.5 [1.7, 3.4]	2.7 [2.2, 3.8]	0.331
Histologic grade G2–G3, no. (%)	31 (43.7)	23 (43.4)	8 (44.4)	0.938
Vascular invasion, no. (%)	29 (40.8)	20 (37.7)	9 (50.0)	0.360
TNM (%)				0.047
T1	10 (14.1)	10 (18.9)	0 (0.0)	
T2–T3	42 (59.2)	32 (60.4)	10 (55.6)	
NA	19 (26.8)	11 (20.8)	8 (44.4)	
Milan = Out (%)	44 (62.0)	36 (67.9)	8 (44.4)	0.076

microvascular invasion. Forty-four patients were classified as beyond the Milan criteria after histological examination of the explanted liver. Clinicopathological data are summarized in Table 2.

During the median follow-up period of 5.26 years, 18 patients (25%) developed intrahepatic and/or extrahepatic HCC recurrence, with most of the lesions already detectable within the first 2 years. Sites of tumor recurrence were liver (2), lung (5), abdominal cavity (4), bone (2), and multiple sites, in addition to the liver (5) [25–27]. No significant difference was found in clinical parameters, except for TNM Stage (Table 2).

#### Association between AI and HCC recurrence

Based on data in the literature [18,20,24], we decided to analyze 19 *loci* situated within or adjacent to specific genes of interest. We found the presence of AI in at least 1 locus in 90% of patients with HCC recurrence compared with 74.5% of patients without recurrence. Significant association was found for D3S2303 ( $p=0.048$ ) considering the presence of LOH (Table 3A),

and D1S407 ( $p=0.006$ ) D9S251 ( $p=0.02$ ), D1S162 ( $p=0.005$ ), D5S592 ( $p=0.005$ ), D9S254 ( $p=0.002$ ) and D10S520 ( $p=0.04$ ) considering high-level LOH (Table 3B).

#### Evaluation of specific panels and association with HCC recurrence

Descriptive analysis of the different panels showed a relevant grade of informativeness in Panel 2 (Table 4). We made a diagnostic test evaluation of each panel to assess the association between AI and the risk of HCC recurrence. No association was found between LOH and HCC recurrence, whereas a significant association was found in all panels considering high-level LOH, and the Panel 2 performance was better than the others (Table 5). The high significance of Panel 2 is further strengthened and supported by FAI analysis, which proves that a high proportion of informative microsatellites exhibit LOH over the total number of informative *loci* in this panel (Table 5).

**Table 3.** Univariate Cox models for microsatellites association with hepatocellular carcinoma recurrence. (A) Patients with loss of heterozygosity (LOH). (B) Patients with high-level loss of heterozygosity.

(A)	exp(coef) [confint]	p	Code	(B)	exp(coef) [confint]	p	Code
D1S407.LOH1	0.74 [0.10, 5.68]	0.7740		D1S407.HighLOH1	49.50 [3.10, 791.37]	0.0058	**
MYCL1.LOH1	1.92 [0.55, 6.70]	0.3036		D3S1539.HighLOH1	1.42 [0.47, 4.33]	0.5329	
D3S1539.LOH1	2.06 [0.81, 5.23]	0.1273		D5S615.HighLOH1	2.06 [0.75, 5.67]	0.1630	
D5S615.LOH1	2.07 [0.77, 5.57]	0.1493		D9S251.HighLOH1	4.58 [1.27, 16.45]	0.0198	*
D9S251.LOH1	2.55 [0.91, 7.19]	0.0763	.	D17S1289.HighLOH1	4.26 [0.93, 19.43]	0.0612	.
D17S1289.LOH1	0.88 [0.25, 3.17]	0.8492		D17S974.HighLOH1	1.31 [0.17, 9.96]	0.7916	
D17S974.LOH1	0.62 [0.08, 4.72]	0.6471		TP53.HighLOH1	10.49 [0.95, 115.73]	0.0550	.
TP53.LOH1	3.11 [0.34, 28.23]	0.3136		D18S814.HighLOH1	2.67 [0.75, 9.51]	0.1286	
D18S814.LOH1	1.46 [0.50, 4.27]	0.4916		D1S162.HighLOH1	31.50 [2.86, 347.37]	0.0049	**
D1S162.LOH1	0.86 [0.25, 2.94]	0.8066		D1S1161.HighLOH1	2.36 [0.68, 8.23]	0.1781	
D1S1161.LOH1	1.00 [0.33, 3.07]	0.9991		D17S786.HighLOH1	8159967418361.53 [0.00, Inf]	0.9999	
D17S516.LOH1	0.00 [0.00, Inf]	0.9988		D5S592.HighLOH1	53.50 [3.35, 855.32]	0.0049	**
D17S786.LOH1	3.40 [0.44, 26.20]	0.2407		D9S254.HighLOH1	8.61 [2.24, 33.07]	0.0017	**
D3S2303.LOH1	6.45 [1.77, 23.55]	0.0048	**	D10S1173.HighLOH1	3.83 [0.50, 29.31]	0.1956	
D5S592.LOH1	3.33 [0.43, 25.79]	0.2485		D10S520.HighLOH1	3.78 [1.07, 13.37]	0.0387	*
D9S254.LOH1	1.77 [0.55, 5.77]	0.3398					
D9S252.LOH1	1.28 [0.29, 5.73]	0.7456					
D10S1173.LOH1	2.52 [0.80, 7.98]	0.1157					
D10S520.LOH1	2.14 [0.61, 7.52]	0.2355					

**Table 4.** Descriptive analysis of microsatellites panels fractional allelic imbalance (FAI) and loss of heterozygosity (LOH).

	Panel 1	Panel 2	Panel 3	Panel 4
n	71	71	71	71
Informativeness				
Median	77.8	93.3	76.9	73.7
IQR	66.7–77.8	86.7–100.0	61.5–76.9	68.4–78.9
Presence of LOH, n (%)	48 (67.6)	51 (71.8)	53 (74.6)	56 (78.9)
FAI				
Mean	0.19	0.13	0.18	0.16
Std. dev.	0.18	0.12	0.15	0.14
Median	0.17	0.12	0.17	0.13
IQR	0.00–0.31	0–0.18	0.04–0.25	0.07–0.21
Range	0.00–0.62	0–0.53	0–0.58	0–0.67
Presence of high-level LOH, no. (%)	31 (43.7)	34 (47.9)	31 (43.7)	34 (47.9)
High-level of FAI				
Mean	0.09	0.05	0.07	0.06
Std. dev.	0.12	0.07	0.10	0.08
Median	0.00	0.00	0.00	0.00
IQR	0–0.14	0–0.07	0–0.11	0–0.08
Range	0–0.5	0–0.30	0–0.42	0–0.40

**Table 5.** Univariate Cox models to assess the association between AI and the risk of HCC recurrence.

At least one microsatellite				
	exp(coef) [confint]	p	Code	Concordance
Panel 1	2.95 [0.86, 10.09]	0.0841	.	0.5961
Panel 2	4.06 [0.94, 17.52]	0.0602	.	0.6092
Panel 3	2.01 [0.59, 6.86]	0.2663	.	0.5552
Panel 4	2.69 [0.62, 11.59]	0.1848	.	0.5683
At least one microsatellite (high-level)				
	exp(coef) [confint]	p	Code	Concordance
Panel 1	2.79 [1.11, 7.01]	0.0286	*	0.6282
Panel 2	4.12 [1.49, 11.35]	0.0062	**	0.672
Panel 3	2.79 [1.11, 7.01]	0.0286	*	0.6282
Panel 4	4.12 [1.49, 11.35]	0.0062	**	0.672
Fractional allelic imbalance				
	exp(coef) [confint]	p	Code	Concordance
Panel 1 FAI	11.64 [1.11, 122.15]	0.0407	*	0.6379
Panel 2 FAI	127.58 [4.60, 3535.44]	0.0042	**	0.6737
Panel 3 FAI	10.04 [0.70, 143.33]	0.0891	.	0.6172
Panel 4 FAI	31.82 [2.08, 487.06]	0.0129	*	0.6627
Fractional allelic imbalance (high-level)				
	exp(coef) [confint]	p	Code	Concordance
Panel 1 FAI	148.58 [9.39, 2351.75]	0.0004	***	0.6825
Panel 2 FAI	12736.03 [192.99, 840485.78]	<0.0001	***	0.7255
Panel 3 FAI	1784.22 [40.74, 78135.33]	0.0001	***	0.6914
Panel 4 FAI	16309.40 [298.76, 890346.50]	<0.0001	***	0.7395

### Association of AI and time to HCC recurrence

After proving the possible association between AI and HCC recurrence, we focused our attention on time to recurrence. Kaplan-Meier curves were performed, proving that LOH, as expected, has no significant correlation with time to recurrence, whereas high-level LOH is strongly correlated with particularly high performance (Figure 1).

### Association of frequency of AI with early HCC recurrence

To further extend our analysis, we assessed whether AI could have a prognostic value in early HCC recurrence detection. Thus, we focused our attention on the 14 patients (11 of whom were classified beyond the Milan criteria after pathology examination) with recurrence within 2 years of LT. We found that the FAI index for high-level LOH has good predictive negative performance. In particular, Panel 2 reaches a negative predictive

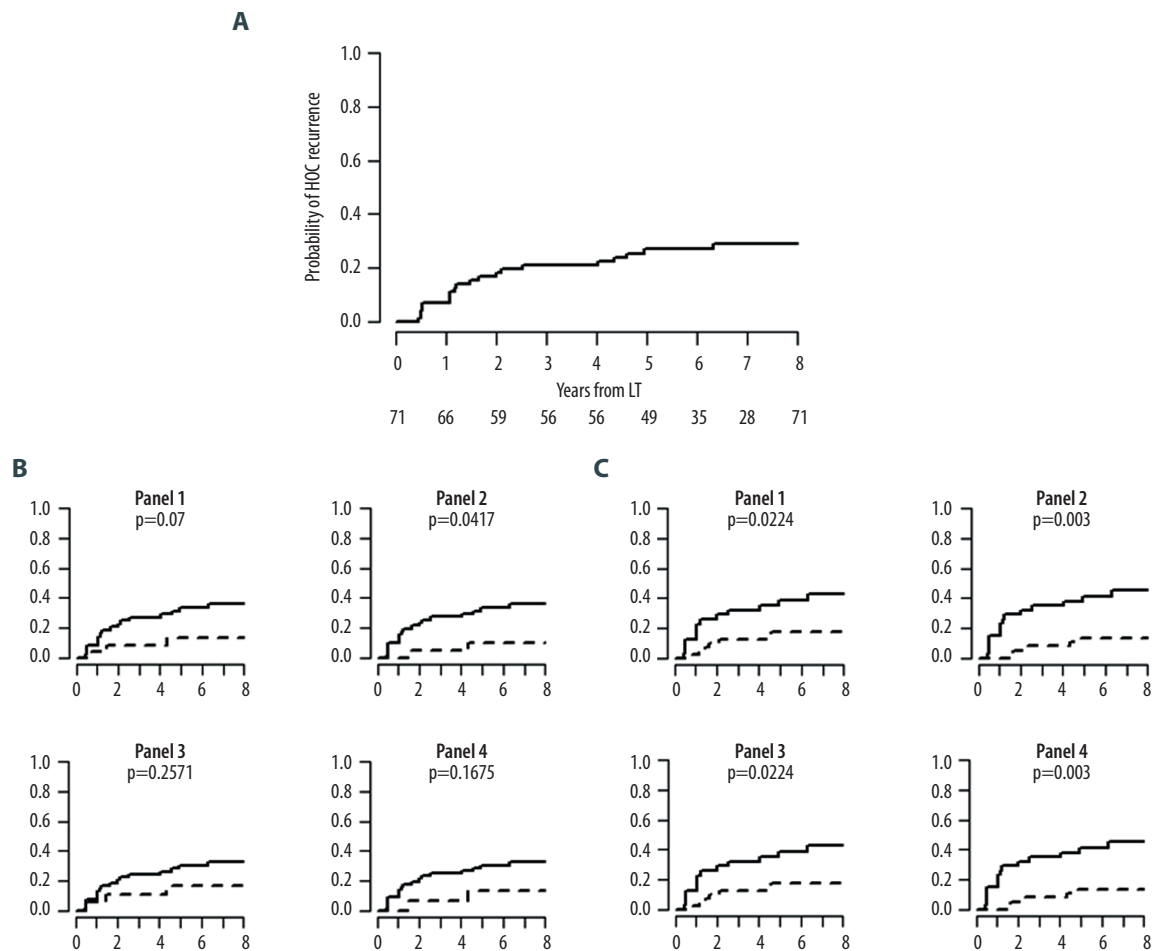
value (NPV) of 95% due to its high sensitivity, with a loss of specificity and positive predictive value (PPV) lower than 35% (Table 6). This was confirmed by receiver operating characteristic (ROC) curve analysis (Figure 2).

### Association of AI with other predictors of HCC recurrence

Finally, we focused our attention on any possible correlation between AI and other clinical or pathological parameters available in our dataset that could be associated with HCC recurrence [5–16]. We evaluated the association between AI and 3 different poor-prognosis HCC indicators: G2/G3 grading, vascular invasion, and Milan criteria. No single microsatellite showed any association with these 3 variables, neither considering presence of LOH nor considering high-level LOH.

As previously reported, 31 patients were graded as having G2/G3 HCC; of these, 18 were graded G2, only 1 was graded G3,





**Figure 1.** Kaplan-Meier time to recurrence curves. (A) Overall study population. (B) Patients with loss of heterozygosis. (C) Patients with high-level loss of heterozygosis.

and the remaining 12 were labelled G2-G3 by the pathologist. Importantly, only 5 of the 13 highest graded (G3+G2-G3) were inside the Milan criteria, and only 1 of these 5 had an early recurrence. Only Panel 2 and Panel 4 succeeded in detecting this recurrence, considering both presence of LOH ( $Sp=0.5$ ,  $Se=1$ ,  $PPV=0.3$ ,  $NPV=1$ ) and high-level LOH ( $Sp=0.75$ ,  $Se=1$ ,  $PPV=0.5$ ,  $NPV=1$ ), thus confirming in this small subset the performance observed in the overall study population.

## Discussion

HCC recurrence after LT is one of the most important causes of mortality and morbidity [28]. At present, there are conflicting recommendations about what tumor characteristics are responsible for HCC recurrence, and the clinical and radiological criteria (e.g., Milan criteria, Up-to-7 criteria) seem to be inadequate because they provide characteristics of the tumor that do not always correspond to the actual pathological

characteristics [16]. Several studies have identified microvascular invasion as the strongest independent predictor factor of recurrence [29,30], associating it with other poor prognostic factors that, except for AFP, can only be known on histological analysis of the explanted liver, or need procedures that are invasive, not easily available, and lack adequate accuracy (e.g., liver biopsy) [31]. Scores based exclusively on radiological or biochemical parameters are not adequate to establish the risk of HCC recurrence, and a number of authors have attempted to combine the 2 methods. Mazzaferro et al. redesigned the Metroticket Paradigm [15], in which patients are assessed on the basis of the combination of AFP levels, and number and size of nodules [32]. A Belgian research group developed a model to predict the likelihood of recurrence based on 4 parameters: Time, Radiological-response, AFP, and INflammation (TRAIN) [33]. Similarly, the MoRAL (Model of Recurrence After Liver transplantation) score combines the biochemical features of AFP and neutrophil-to-lymphocyte ratio with the radiological feature of tumor dimension (tumor

**Table 6.** Predictive performances of early recurrence panels.

Loss of heterozygosis					
	Se	Sp	PPV	NPV	Acc
Panel 1 FAI	0.8462	0.3621	0.2292	0.913	0.4507
Panel 2 FAI	0.9231	0.3276	0.2353	0.95	0.4366
Panel 3 FAI	0.8462	0.2759	0.2075	0.8889	0.3803
Panel 4 FAI	0.9231	0.2414	0.2143	0.9333	0.3662
High-level loss of heterozygosis					
	Se	Sp	PPV	NPV	Acc
Panel 1 FAI	0.6923	0.6207	0.2903	0.9	0.6338
Panel 2 FAI	0.8462	0.6034	0.3235	0.9459	0.6479
Panel 3 FAI	0.6923	0.6207	0.2903	0.9	0.6338
Panel 4 FAI	0.8462	0.6034	0.3235	0.9459	0.6479

size  $\geq 3$  cm) [34]. In the present retrospective study, we evaluated the relationship between prognostic clinicopathological factors and HCC recurrence, but no statistically significant association was found except for TNM stage (Table 2;  $p=0.047$ ).

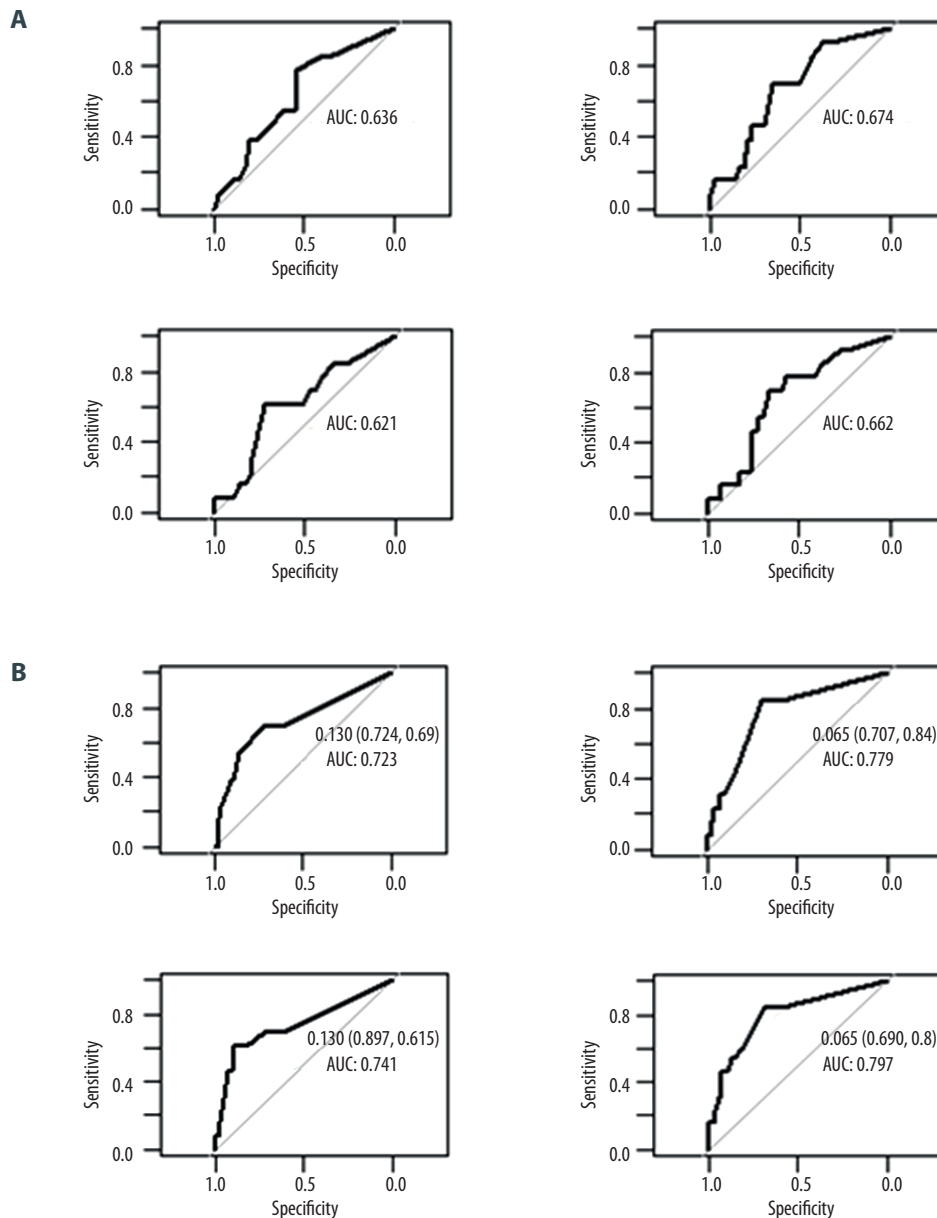
To date, however, it has not been possible to identify sufficiently reliable markers of the biological behavior of HCC. Therefore, the attention of researchers is increasingly shifting from the analysis of clinical and radiological characteristics to the analysis of genetic mutations typical of this neoplasm. In this context, several studies in the last few years have mentioned AI (LOH) as a possible predictor of HCC recurrence [18,20,21,24]. Marsh et al. developed a multivariate model (artificial neural network – ANN) based on 5 risk factors (sex, tumor number, tumor size, lobar tumor distribution, and grade of vascular invasion) that could predict the risk of tumor recurrence [35]. This model, in association with the study of AI based on tissue microdissection genotyping in 9 microsatellites at 6 genomic *loci* [18,20], had an accuracy of 100%, with a discriminatory power of 85% in the predicted 3-year recurrence outcome [36]. In addition, a recent meta-analysis of 41 eligible studies noted the importance of identifying serological (pre-LT AFP and pre-LT DCP) and molecular biomarkers able to predict HCC recurrence [37]. In our study, we found a significant association between AI in specific microsatellite *loci* and the risk of HCC recurrence, validating for the first time in a European population the results of several other studies [18,20,22,24,35]. A statistically significant association with tumor recurrence was found for D3S2303 (gene OGG1;  $p=0.048$ ) considering the presence of LOH (Table 3A), and D1S407 (gene CMM;  $p=0.006$ ) D9S251 (gene CDKN2A/p16;  $p=0.02$ ), D1S162 (gene L-myc;  $p=0.005$ ), D5S592 (gene MCC;  $p=0.005$ ), D9S254 (gene CDKN2A/p16;  $p=0.002$ ), and D10S520 (gene

PTEN;  $p=0.04$ ) considering high-level LOH. Thus, we confirmed that the LOH frequency on 9p21 locus (D9S251 and D9S254, *CDKN2A* gene) is associated with recurrence [21].

Many retrospective studies have used different and arbitrary cumulative mutational damage indexes (e.g., FAI) to investigate the relationship between AI and HCC recurrence, and identified specific discriminatory values between true positives and false positives using ROC curve analysis constructed using results obtained in each study [18,20–23]. Our data show that the FAI index for high-level LOH in a specific panel of microsatellites (Panel 2) has a good negative predictive performance, with a 95% NPV, to identify early-HCC recurrence. Unfortunately, the PPV for each analyzed panel cannot be considered satisfactory. Thus, our analysis confirms the existence of an association between HCC recurrence and LOH in specific *loci* (specifically high-level LOH), proving at the same time that AI should not be considered as a positive predictor of tumor recurrence, but its absence can be considered as a negative one.

Of note, in contrast to previously research, our study did not analyze the AI between neoplasia and healthy fragments of liver parenchyma, but obtained similar results by comparing very limited quantities of neoplastic tissue with whole blood, thus reducing the potential invasiveness of the analysis. In fact, previous studies have indicated that the use of minimally invasive laparoscopic techniques allows the successful resection of HCC nodules without increasing the risk of hepatic failure or peri-operative complications [38,39]. Thus, such surgical approaches could be proposed not only as a bridge-therapy for LT, but also as a useful diagnostic tool to evaluate AI between neoplastic tissue and whole blood [40]. The scenarios that would lead to a similar combined





**Figure 2.** ROC curve for prediction of early recurrence. **(A)** Patients with loss of heterozygosis. **(B)** Patients with high-level loss of heterozygosis.

use of minimally invasive surgery and AI to predict patients at greater risk of recurrence could change the priorities in organ allocation procedures and would ensure tailored post-transplant therapies (e.g., changes of immunosuppression regimens).

Our study has several limitations. This was an analysis with a limited number of patients, and we could not evaluate the effect of pre-transplantation treatments on survival and recurrence (e.g., radiofrequency thermal ablation and trans-arterial chemoembolization). Unfortunately, there was no donor and recipient allelotyping, and we could not distinguish whether

the tumor recurrence represents metastasis of the first cancer or a donor-derived *de novo* cancer, as proposed by recent studies [25–27]. Moreover, HCC genotyping can be complicated by interpretative difficulties due to heterogeneity of tumor tissues from a pathological specimen containing various subpopulations of cells. Unfortunately, we could not perform microdissection, and DNA from nonmalignant cells could have contaminated the DNA extracted from these samples. Finally, working with FFPE tissues of the native liver, and DNA extraction and amplification might be affected by tissue fixation time and progressive DNA degradation in archival tissue blocks.

In the post-LT setting, FAI could provide useful information to adapt and personalize therapies (e.g., immunosuppression protocols), thus reducing the risk of recurrence. However, the application of molecular markers needs further evaluation before being used as selection criteria for LT. We intend to validate the HCC recurrence prediction model based on the use of specific microsatellites (especially D9S251) in a future study in order to verify its concrete predictive ability and to identify patients at lower risk of HCC recurrence, combining clinicopathological, radiological, and genomic data, easily available in the pre-LT setting. Moreover, using a larger number of patients with more complete clinical information, we intend to study the association of AI in each locus with clinicopathological characteristics and to analyze its biological role in HCC development, progression, and aggressiveness.

## Conclusions

One of the important ethical implications of LT for HCC, which is the subject of lively debate in the transplantation community and beyond, is that ability to predict which recipients will

successfully complete the post-transplant course. Transparency and clinical clarity are 2 of the cornerstones in sharing experiences with the medical community at large. We observed that the FAI for a high-level LOH has a good NPV for tumor recurrence within 2 years after transplant (95%). These data confirm a relevant role of the *CDKN2A* gene for HCC recurrence. Our data suggest that the information obtained by AI analysis can be useful, and has a prognostic application in risk management of HCC recurrence in patients who have undergone LT, especially in early tumor recurrence. One of the important ethical implications of LT for HCC, which is the subject of lively debate in the transplantation community and beyond, is the ability to predict which recipients will successfully complete the post-transplant course. Developing new protocols for immunosuppressive regimen and surgical decision-making is paramount for continued success in this delicate field of medicine.

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