Extracellular vesicles as potential biomarkers for diagnosis and recurrence detection of hepatocellular carcinoma: A pilot study

Mazen A. Juratli

Mazen.juratli@ukmuenster.de

Muenster University Hospital, Muenster University

Nicola S. Roushansarai

Muenster University Hospital, Muenster University

Elsie Oppermann

Frankfurt University Hospital, Goethe University

Dhruvajyoti Roy

Helio Health

Andreas Schnitzbauer

Frankfurt University Hospital, Goethe University

Andreas Andreou

Muenster University Hospital, Muenster University

Philipp Houben

Muenster University Hospital, Muenster University

Shadi Katou

Muenster University Hospital, Muenster University

Felix Becker

Muenster University Hospital, Muenster University

Jens Peter Hoelzen

Muenster University Hospital, Muenster University

Andreas Pascher

Muenster University Hospital, Muenster University

Wolf O. Bechstein

Frankfurt University Hospital, Goethe University

Benjamin Struecker

Muenster University Hospital, Muenster University

Thomas Vogl

Department of diagnostic and interventional Radiology, Frankfurt University Hospital, Goethe University

Sabine Michalik

Department of diagnostic and interventional Radiology, Frankfurt University Hospital, Goethe University

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Abstract

Hepatocellular carcinoma (HCC) is the most common primary malignant liver tumor and a leading cause of cancer-related deaths worldwide. However, current diagnostic tools are often invasive and technically limited. Extracellular vesicles (EVs), which play vital roles in HCC growth and metastasis, serving as potential novel biomarkers. Molecular analysis of peripherally EVs could be revolutionary for early tumor diagnosis and detecting tumor recurrence. A prospective single-center cohort study including 37 HCC patients and 20 patients with non-malignant liver disease (NMLD), as a control group, was conducted. Peripherally EVs of both groups were analyzed before and after liver surgery. The study utilized microbead-based magnetic particle sorting and flow cytometry to detect 37 characteristic surface proteins of EVs. Furthermore, HCC patients who experienced tumor recurrence (R-HCC) within 12 months after surgery were compared to HCC patients without recurrence (NR-HCC). R-HCC patients (n = 12/20) showed significantly lower levels of CD31 compared to NR-HCC patients ($p = 0.0033$). The NMLD-group showed significantly higher expressions of CD41b than the HCC group ($p = 0.0286$). The study determined significant short-term changes in CD19 dynamics in the NMLD-group, with preoperative values being significantly higher than postoperative values ($p = 0.0065$). This finding of our pilot study suggests EVs as potential targets for the development of diagnostic and therapeutic approaches for the early and non-invasive detection of HCC recurrence by monitoring the dynamics of specific EVs markers, such as CD41b and CD31, in HCC patients. Overall, the potential of EVs as diagnostic and therapeutic targets for HCC recurrence represents a promising area of research that could have a significant impact on the management of this disease in the future.

Introduction

Hepatocellular carcinoma (HCC) is the most common primary liver cancer, with an increasing incidence and high recurrence rate, which negatively impacts prognosis and overall survival [1]. Currently, hepatic resection, radiofrequency ablation, and liver transplantation represent curative therapeutic options for HCC at early-stages. Recurrence rates of early-stage HCC remain high by 35% within the first year [2] and resemble a crucial prognostic factor since early recurrence is associated with developing incurable disease [3, 4]. The liver represent the most frequent site of HCC recurrence, lately reviewed by 66% [2], leading to the question, whether therapeutic options can be optimized. Currently available diagnostics include radiographic differentiation of indeterminate or hazardous lesions, and alpha-fetoprotein level measurement, which remain limited regarding the analysis of tumor biology [5].

Molecular profiling and immune oncologic risk factors could lead to earlier detection of HCC recurrence at a stage, where curative resection and ablation modalities are amenable [5]. Hence, innovative early biomarkers to detect micro metastases or recurrence initiating immunomodulation are awaited to improve curative therapeutic options in early recurrence after HCC resection.

Extracellular vesicles (EVs), which play a crucial role in cellular communication, metastasis, and immune tolerance of HCC [6, 7], could enhance early recurrence diagnosis and thus optimize HCC treatment. EVs

are a heterogenous population of particles that consist of lipid-bilayer membrane-coated vesicles ranging from 50 to 140 nm in size [6]. EVs cargo consist of miRNAs, RNAs, lipids and proteins [4]. The composition can be altered due to external stimuli such as the blood-ph and hypoxia [4]. Regarding systemic and local tumor dynamics, it was recently shown, that hepatic tumors prepare a comforting microenvironment via release of EVs, initiate tumor angiogenesis and even might evade the immune cell recognition through EV mediated mechanisms [8]. The aims of this study is to characterize the dynamics of EV surface markers in recurrent and non-recurrent HCC patients up to 12 months after surgery. Patients with non-malignant liver disease (NMLD) were used as controls.

Material and methods

Patient characteristics and sample collection

The prospective single-center cohort study was conducted at the Johann Wolfgang Goethe University Frankfurt between 2016 and 2019. This study was approved by the ethics committee of the University of Frankfurt (approval number: 321/16). Informed consent forms were signed and obtained from all patients. All experiments were performed in accordance with relevant guidelines and regulations. Blood samples were collected from 36 patients with HCC and 20 patients with non-malignant liver disease (NMLD, control group) before and after surgery. After the follow up period of 12 months, 20 HCC patients remained for blood sample retrieval and recurrence analysis. Overall, 17 patients were excluded after 12 months due to loss of follow up and incomplete data set. Out of these 20 patients, 12 (60%) patients were diagnosed with recurrent HCC after a median follow-up period of 12 months.

Isolation of EVs

Highly purified EVs were isolated by using the Isolation Kit Pan Human (Miltenyi Biotec, Bergisch Gladbach, DE). Serum EV surface expression was analysed by a bead based multiplex EV analysis and flow cytometry (MACSPlex Exosome Kit, human, Miltenyi Biotec, Bergisch Gladbach, DE). Detection of 37 EV surface epitopes, excluding two negative controls (Table 1) was conducted according to manufacturer’s instructions. The antibody coated beads capture serum EVs which express the corresponding molecule. Next flow cytometric based quantification was conducted using allophycocyanin (APC) conjugated CD9, CD63 and CD81 for detection of antibody coated bead-EV complexes Fig. 1.

Table 1
Surface epitopes:

CD3	CD1c	CD63	CD42a	CD142
CD4	CD25	CD40	CD24	CD45
CD19	CD49e	CD62P	CD86	CD31
CD8	ROR1	CD11c	CD44	CD20
HLA_DRDPDG	CD209	CD81	CD326	CD14
CD56	CD9	MCSP	CD133	
CD105	SSEA4	CD146	CD29	
CD2	HLA_ABC	CD41b	CD69	

Flow cytometry

Measurement of 37 surface proteins was achieved using BD FACSCanto II and analysed by FACSDiva (Becton Dickinson, USA). The median APC fluorescence of each specific population was normalized according to detection antibodies. Surface protein expression was measured as fluorescence intensity, considering the corresponding control antibody as measurement threshold and protein expression below the threshold as negative.

Statistical analysis

Statistical analysis was performed with SPSS (IBM SPSS Statistics, New York, USA) and GraphPad (GraphPad Prism 8.0.0, San Diego, USA for graph creation. All data showed asymmetric distribution. Statistical analysis included Mann Whitney U test for patient characteristics. Analysis of protein expression was conducted by Friedman test for dependent and Kruskal Wallis test for inter group comparisons plus Bonferroni post hoc test. Results were visualized by median and 95% confidence interval. A level of $p < 0.05$ was considered statistically significant.

Results

Patients characteristics

The HCC group, which included all HCC patients, had a higher age (64.05 ± 10.20 year) and a higher percentage of males (63.64%) than patients with NMLD, as shown in Table 2. Notably, patients in the NR-HCC group were older than those in the R-HCC group. The R-HCC and NR-HCC groups had similar gender distribution and non-alcoholic steatohepatitis (NASH) rates. The R-HCC group had a lower fibrosis rate, a higher incidence of hepatic cirrhosis, a slightly higher rate of open surgery, and smaller tumor volume compared to the NR-HCC group, as demonstrated in Table 2.

Table 2
Characteristics of HCC, NMLD, R-HCC and NR-HCC patients

Characteristics	HCC (n = 36)	NMLD (n = 20)	p Value HCC vs NMLD	R-HCC (n = 12/20)	NR-HCC (n = 8/20)	p Value R-HCC vs NR-HCC
Age (M ± SD)	64.85 ± 11.64	48.40 ± 14.13	< 0.0001	61.88 ± 10.66	67.31 ± 8.48	0.31
Sex (n% males)	58.33%	15.0%	0.0006	75.00%	75.00%	> 0.99
Liver fibrosis	50.0%	-		58.33%	37.50%	0.65
Liver cirrhosis	38.89%	-		25.00%	62.50%	0.17
NASH	22.22%	-		16.67%	12.50%	> 0.99
Type of surgery (n% open surgery)	66.67%	65.0%	0.31	83.33%	75.00%	> 0.99
Tumor volume (cm^3) (MW ± SD)	187.71 ± 247.15	-		183.86 ± 143.97	211.70 ± 243.83	0.85
R Status (% R0)	R0 = 86.11%	-		R0 = 66.67%	R0 = 87.5%	> 0.99
AFP before surgery (ng/ml; M ± SD)	3045.72 ± 11022.15	2.23 ± 1.05	< 0.0001	3694.49 ± 13093.02	2163.93 ± 6475.52	0.78
AFP after surgery (ng/ml; M ± SD)	3103.89 ± 10827.33	2.14 ± 1.10	< 0.0001	4082.81 ± 13635.75	1888.33 ± 4539.59	0.74

M = Mean; SD = standard deviation; NASH = non alcoholic steatohepatitis; after surgery = within the first week after surgery

EVs dynamics in NMLD patients

In the NMLD patients, the expression of CD19 was significantly upregulated postoperatively (p = 0.0065; Fig. 2A). Additionally, the NMLD patients showed increased protein expressions of MSCP and CD62P postoperatively compared to preoperatively (p = ns).

EVs dynamics in HCC patients

The short-term expression of EV surface proteins in the HCC group remained stable from pre- to postoperative with no significant differences. However, protein expressions of MSCP and CD62P were decreased pre- to postoperative with no significant differences (Fig. 2B). Regarding the long-term comparison of the R-HCC and NR-HCC group (n = 20), CD31 expression was significantly higher in the NR-HCC group than in the R-HCC group up to 12 months after surgery (p = 0.0031; Fig. 3A). Interestingly, the NR-HCC group showed higher expressions of CD9, CD62P, CD42a and CD29 up to 12 months postoperative compared to the R-HCC group, but these differences were not statistically significant (Fig. 3).

Comparision of EVs dynamics between HCC and NMLD patients

The HCC group showed a significantly decreased expression of CD41b compared to the NMLD group (p = 0.0286), but there were no significant differences in combined pre and postoperative EV expression between the two groups. Interestingly, both the HCC and NMLD groups exhibited high expressions of CD9, CD41b, CD42a, CD29 and CD31 (Fig. 3B).

Discussions

In recent years liquid biopsy has shown promise as a diagnostic tool for HCC and the prediction of its recurrence [9–11]. While recent studies have focused on circulating tumor DNAs and micro RNAs [12], this pilot study aimed to enable further evaluation of EV surface protein expressions as novel biomarkers.

Recently, surface markers of EVs have gained attention since they indicated presence of HCC and cholangiocarcinoma with increased levels of CD147 and CD133 [13]. Additionally, EVs transfer proteins and different types of RNAs, including miRNAs, from highly malignant cells to surrounding cells, promoting HCC migratory and metastatic capacities [14]. This is of particular interest because tumor invasion lead to early recurrence of HCC after resection, resulting in high recurrence rates [15]. Advances in this field may lead to earlier diagnosis and more efficient targeted therapies for HCC. Recent investigations, from Sun et al. (2020) showed promising results regarding HCC specific EV purification [16].

In our project, EV surface protein expressions were analysed up to 12 months for the evaluation of EV expression dynamics and the compared groups showed similar clinical characteristics. We aimed to analyse EV expressions before surgery and to determine the surgical effect on the EVs short- and long-term dynamic. One of the major challenges in HCC is that no specific markers for predicting HCC recurrence were found. This pilot study identified differences in the EV surface protein patterns of HCC patients, in general, and HCC patients with recurrence and non-recurrence, for the first time.

The underlying results could facilitate further investigations on combined HCC specific and systemic immune responses regarding HCC and recurrent HCC. This could enable the identification of a suitable biomarker in future investigations.

In the NMLD group, the surgical procedure led to a significant increase in the CD19 rate postoperatively, which is consistent with current literature, as CD19 increment has already been reported in benign hepatic surgery [17]. CD19 is a lymphocyte marker expressed in sterile inflammation, such as after surgical trauma.

Interestingly, no significant changes in the EVs dynamics were observed due to the surgical resection of the liver in the HCC group. However, twelve months after surgery, a significant negative association between CD31 and HCC recurrence was found in the underlying pilot study. CD31 is a leuko-endothelial marker expressed on tumor cells and has been found to promote HCC metastasis [18–20]. However, CD31 has also been associated with cirrhosis [11], which is consistent with the underlying results and a higher cirrhosis rate in the NR-HCC group compared to the R-HCC group. We strongly suggest re-evaluating the relevance of CD31 in HCC recurrence in a larger study.

The surface protein CD41b resembles a platelet-derived exosome marker recently found to be increased in nonalcoholic fatty liver disease (NAFLD), which could explain the significant increment of CD41b in the NMLD group compared to the HCC group in this study. Evidence can be found for CD41-positive EVs in alcoholic liver disease [1]. Regardless of the consistency with other studies, the underlying findings on CD41b should be taken with caution since this marker was reported as unstable in LB [1, 17]. One limitation or this conducted study is the small number of patients. However, the underlying results could lay the foundation for individualized and efficient HCC diagnostics to optimize tumor treatment.

Conclusions

The underlying study investigated EV surface patterns in HCC and NMLD. In summary, we have demonstrated a straightforward method for isolating EVs, highlighting differences in EV surface expressions between HCC patients and NMLD patients. As pointed out, some markers i.e., CD41b, seem to play a role in HCC diagnostics and CD31 may be used as recurrence marker. These results aid further investigation on EV profiling in HCC recurrence which might enable the identification of suitable biomarker and further optimized, individualized therapy in the future.

Declarations

Funding details

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Conflict of Interest statement

M.A.J., N.R., E.O., D.R., A.S., A.A., S.M., T.V., P.H., S.K., F.B., J.H., A.P., W.O.B., B.S. declare no conflicts of interest. The authors have read the journal's policy and have the following competing interests: DR is an employee of Laboratory for Helio Health, Inc. This does not alter our adherence to Scientific reports policies on sharing data and materials. There are no patents, products in development or marketed products associated with this research to declare.

Data avaibilitiy

The data that support the findings of this study are available from the corresponding author, [MAJ], upon reasonable request.

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Figures

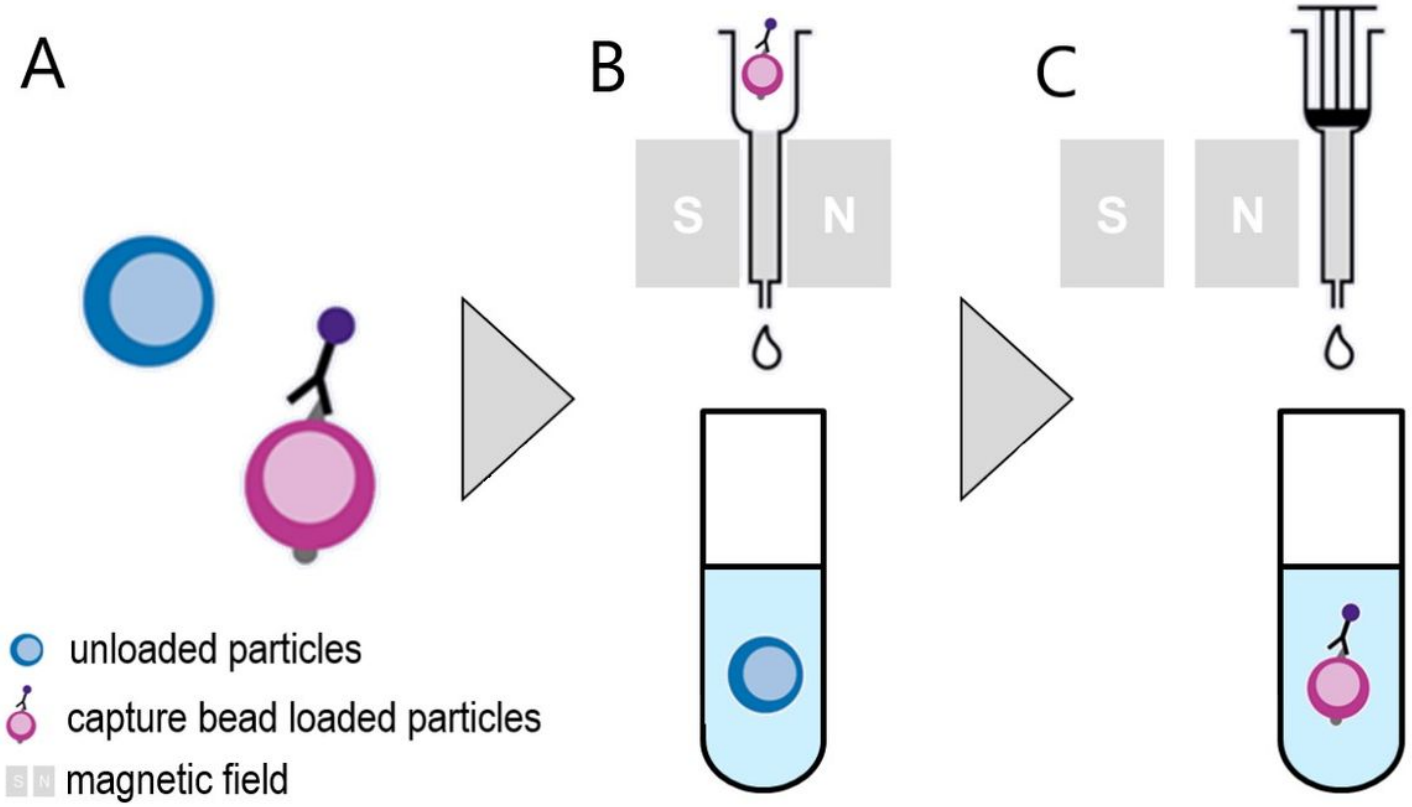

Figure 1

Bead-based magnetic adsorption of EVs.

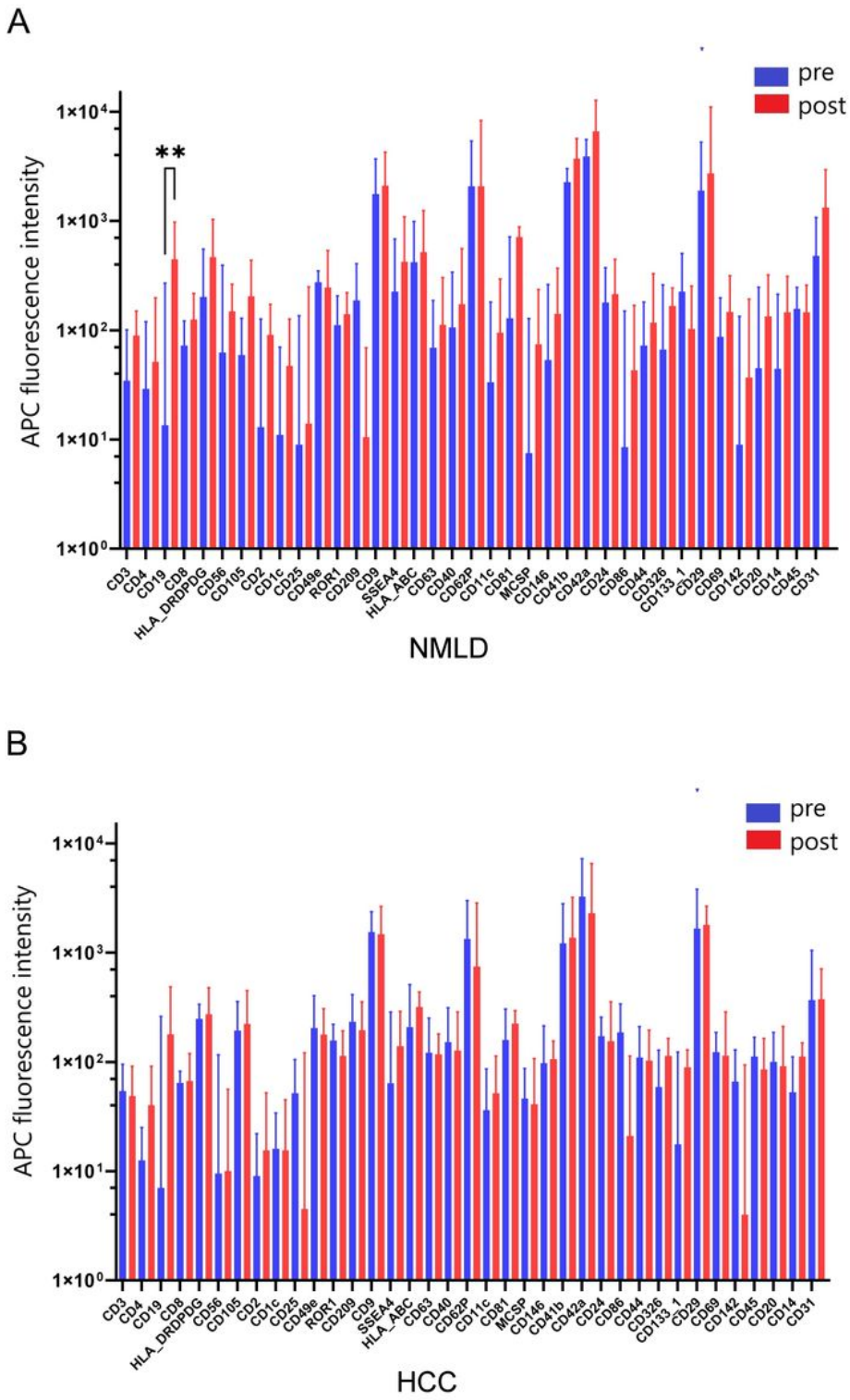

Figure 2

Surface protein expression of EVs in the NMLD group (A) and HCC group (B) for preoperative and postoperative probes in blue and red, respectively. In the NMLD patients, median values of APC fluorescence intensities show increased protein expressions of MSCP and CD62P postoperatively compared to preoperatively. CD19 expression is postoperatively significantly increased in the NMLD

group (p=0.0065). In the HCC group, protein expressions of MSCP and CD62P were decreased pre- to postoperative with no significant differences.

NMLD: Non-malignant liver disease. HCC: Hepatocellular carcinoma.

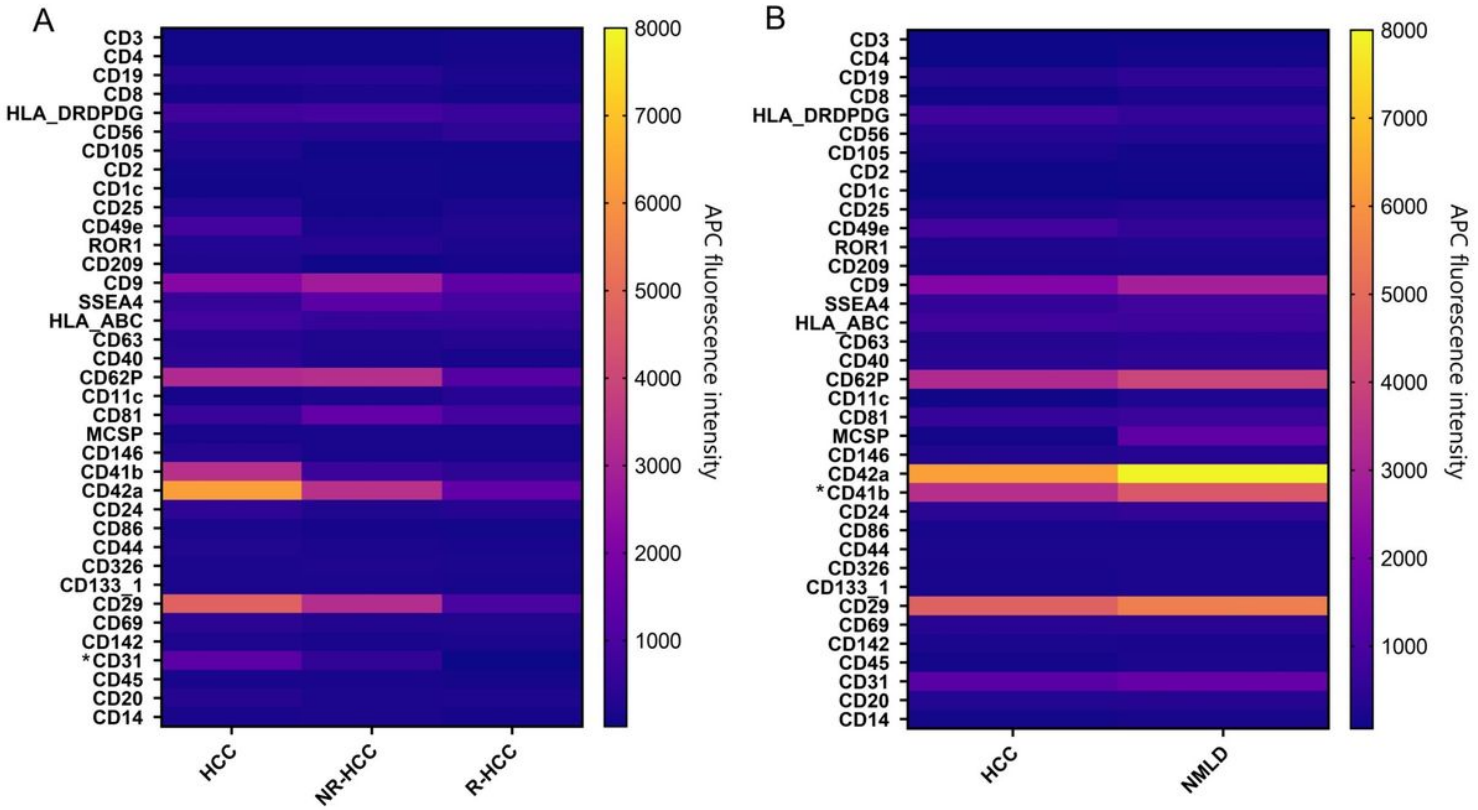

Figure 3

(A) Development of EV expression in the R-HCC and the NR-HCC group up to 12 months postoperative. CD31 expression was significantly higher in the NR-HCC group than in the R-HCC group up to 12 months after surgery (p= 0.0031).(B) Comparison of pre- and postoperative EV expression between the HCC and the NMLD group. The HCC group showed a significantly decreased expression of CD41b compared to the NMLD group (p= 0.0286).

NMLD: Non-malignant liver disease. R-HCC: Recurrence hepatocellular carcinoma. NR-HCC: Non-recurrence hepatocellular carcinoma.