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Original Article

Low adipocyte hepatocellular carcinoma is associated with aggressive cancer biology and with worse survival

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Abstract: Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related death worldwide, and non-alcoholic fatty liver disease is strongly associated with its development. To explore the role of adipocytes in HCC, we investigated intratumoral adipocytes, also known as cancer-associated adipocytes (CAA). Based on our prior breast cancer findings, we hypothesized that low intratumoral adipocytes would be associated with aggressive cancer biology, worse tumor microenvironment (TME), and clinical outcomes. The Cancer Genome Atlas (TCGA) was used and validated by the Gene Expression Omnibus (GEO) cohort. xCell algorithm was used to quantify intratumoral adipocytes and top 90% were defined as adipocyte high (AH) and bottom 10% as adipocyte low (AL). We found that AL-HCC was significantly associated with worse disease-free survival (DFS), disease-specific survival (DSS), and overall survival (OS). AL-HCC were higher-grade, had high MKI67 expression, enriched cell proliferation-related gene sets, and had increased altered fraction, aneuploidy, and homologous recombination defects. Also, anti-cancer immune cells, CD8, Th1, and M1 cells, as well as pro-cancer Th2 cells were increased in AL-HCC. Micro-RNAs miR-122 (associated with cholesterol metabolism) and miR-885 (associated with liver pathologies) were significantly increased in the AL TME. In conclusion, we found that AL-HCC has worse patient outcomes and is biologically more aggressive with enhanced cell proliferation. Our findings take initial steps to clarify the role of adipocytes in HCC.

Keywords: Hepatocellular carcinoma, HCC, non-alcoholic steatohepatitis, NASH, non-alcoholic fatty liver disease, NAFLD, adipocytes, intratumoral adipocytes, cancer associated adipocytes, CAA

Introduction

Hepatocellular carcinoma (HCC) is globally the sixth most common cancer, but it ranks the second leading cause of cancer-related death worldwide due to its aggressiveness and late diagnosis [1]. Inherited, environmental, and infectious etiologies have been classically identified as the underlying cause of hepatocyte malfunction leading to oncogenesis [2]. These etiologies, especially alcohol intake and hepatitis viruses, remain responsible for a significant portion of the incidence of HCC [3]. On the other hand, 30-40% of patients do not have any of these identified major risk factors [4]. Non-alcoholic fatty liver disease (NAFLD), including non-alcoholic steatohepatitis (NASH), has been proposed as a widespread underlying factor for idiopathic HCC [5]. However, there is primarily indirect evidence for the causation between NAFLD and HCC [6].

Adipocytes are one of the stromal cells that constitute the tumor microenvironment (TME) that is known to play critical roles in cancer progression and metastasis. Long thought to be indolent bystanders, their part in usurping usual roles and machinery to grow and spread

cancers is being uncovered in various malignancies metastasis [7-16]. Our group's prior work in breast cancer shows that cancer-associated adipocytes (CAA) interact with cancer cells and promote proinflammatory cytokine secretion, contributing to pro-cancer inflammation and cancer progression [15, 17]. We also showed that the malignancies with the highest overall infiltration of CAA were breast and liver cancer [15], thus CAA are of particular interest for their role in HCC. Interestingly, we further found in breast cancer that a high CAA density was associated with a favorable TME, and low CAA density was associated with enhanced cell proliferation and with advanced grade tumors [15]. Based upon these findings, we hypothesized that low intratumoral density of CAA is associated with more aggressive cancer biology and with poorer patient outcomes.

In order to investigate the association between CAA infiltration and HCC progression, we estimated the intratumoral infiltration of CAA utilizing xCell, a bioinformatic algorithm that identify adipocytes by gene expression profile [18]. Each cohort was divided into intratumoral adipocyte-low (AL - bottom 10%) and intratumoral adipocyte-high (AH - top 90%) groups. Using these defined cohorts, we assessed patient outcomes and compared the active gene sets and the TME using in silico analysis of publicly available databases of human HCC tumors.

Materials and methods

Clinical and transcriptomic data acquisition of HCC patients

Clinicopathologic and gene expression data from The Cancer Genome Atlas (TCGA) was obtained through cBioPortal as previously described by our group [19-35]. This was compared to datasets of HCC subjects identified from the Gene Expression Omnibus (GEO) [36]. We used a computational algorithm, xCell, previously used by our group to quantify intratumoral adipocytes across the publicly available HCC transcriptomes [15, 24, 26-28, 37-42]. The density of the adipocytes within the tumor volume were calculated. These densities were then used to define the cohorts: up to the 10th percentile were defined as adipocyte low (AL) and the remainder were defined as adipocyte high (AH).

Tumor microenvironment

The tumor microenvironment was assessed utilizing a computational algorithm, xCell, mentioned above. The infiltration of pro-cancer immune cells as well as anti-cancer immune cells were estimated using this algorithm. The scores of fraction altered, aneuploidy score, homologous recombination defects, and number of segments were calculated using the report by Thorsson et al. [43].

Grading, staging, and outcomes

The AH and AL cohorts were assessed and compared for their corresponding clinicopathologic data. Grading and staging data respectively were distributed across 4-tier systems utilized by TCGA and GEO. Clinical outcomes assessed included disease-free survival (DFS), disease-specific survival (DSS), and overall survival (OS), which were available in TCGA.

Gene set enrichment analysis (GSEA)

We utilized a gene set enrichment assay (GSEA) to determine the most active biologic functions within the adipocyte high and low tumors [44]. Gene set enrichment analysis software (version 4.0, Broad Institute, Cambridge, MA, USA) was used.

Statistical analysis

All statistical analysis was conducted using R software (v 4.0.2). Survival analysis was conducted using Kaplan-Meier survival curves using greyzoneSurv packages. One-way ANOVA or Fisher's exact test was used to assess the differences between groups with a p-value < 0.05 considered statistically significant. Spearman correlation coefficient was used for the association between adipocyte density and MKI67 expression. Boxplots were of the Tukey type depicting medians and interquartile ranges.

Institutional review board statement

Ethical review and approval were waived for this study due to using human tumor data that is publicly available and de-identified.

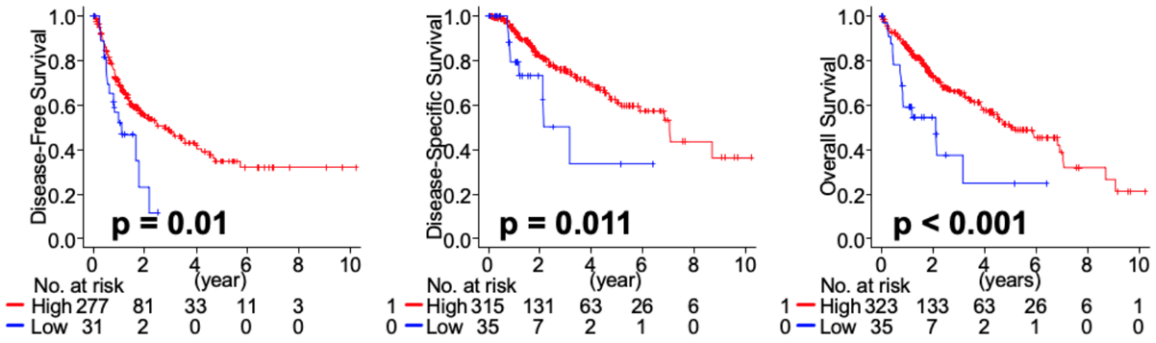

Figure 1. Kaplan Meier survival analysis (disease-free survival (DFS), disease-specific survival (DSS), and overall survival (OS)) of adipocyte high (AH - red line) and adipocyte low (AL - blue line) in the TCGA liver cancer cohort. The number of patients at risk is shown below the X-axis of each panel. *p*-value < 0.05 was considered statistically significant.

Results

There was a significant difference between adipocyte-high (AH) and adipocyte-low (AL) HCC in disease-free survival (DFS), disease-specific survival (DSS), and overall survival (OS)

First, we wanted to assess whether CAA infiltration was associated with any difference in HCC survival outcomes. Based on our prior work, we hypothesized that AL would be associated with worse patient outcomes than AH liver cancers. Our analysis confirmed our suspicion; when the TCGA HCC cohort was divided into adipocyte high (AH) and low CAA (AL), there was a significant difference in disease-free survival (P = 0.01), disease-specific survival (P = 0.011), and overall survival (P < 0.001) (**Figure 1**).

AL tumors were significantly associated with higher-grade tumors and increased MKI67 activity

Since AL tumors were associated with poorer patient outcomes, we wanted to explore the underlying biology of these results. To this end, we evaluated for differences of adipocyte infiltration in the following: staging (I-IV) as an indication of tumor aggressiveness toward the external environment, grade (1-4 in TCGA, 1-3 in GSE89377) as a pathological determination of cancer cell proliferation, and MKI67 expression, which is a molecular biological parameter of cancer cell proliferation [15]. The results obtained in TCGA cohort were validated with another HCC cohort from the Gene Expression Omnibus (GEO), GSE76427. In terms of staging, we found a tendency in both TCGA and GEO cohorts that AL HCC were higher stage tumors. However, this did not reach statistical significance and was less accurate for the most advanced stage IV cancers due to small sample size (**Figure 2A**). Concerning grade, AL HCC were significantly associated with higher grades compared to AH HCC in both TCGA and GEO cohorts (**Figure 2B**; TCGA P < 0.001, GEO p = 0.013). Finally, concerning MKI67, as we expected, AL HCC were found to have significantly increased MKI67 expression in both TCGA and GEO cohorts (**Figure 2C**; P < 0.001 and P < 0.001). Additionally, the total density of HCC intratumoral adipocytes was significantly inversely correlated with MKI67 activity consistently in both cohorts (**Figure 2C**; r = -0.287 and r = -0.369, respectively).

AL HCC were significantly associated with cell-cycle and cell proliferation-related gene sets

We next investigated which gene sets were enriched with AL HCC since these cancers were higher grade and more aggressive. To assess this, we conducted gene set enrichment analysis (GSEA) on the AL HCC in the TCGA and GEO cohorts. We found significant enrichment of all the cell proliferation-related gene sets in Hallmark collection; E2F targets, G2M checkpoint, mitotic spindle, MYC targets V1, and MYC targets V2 In the TCGA cohort (**Figure 3**). Similarly, all the cell proliferation gene sets exept MYC Targets V2; E2F targets, G2M checkpoint, mitotic spindle, and MYC targets V1, were all significantly enriched in GSE76427 (**Figure 3**). Based upon these results, we determined that AL is associated with increased cell proliferation.

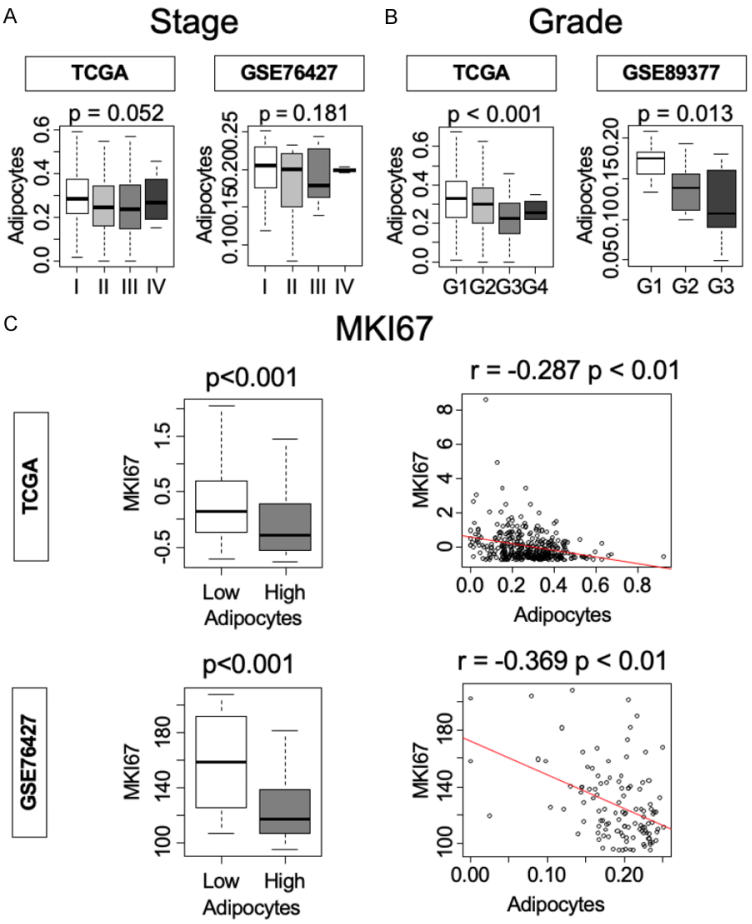

Figure 2. The stage, grade, and MKI67 expression in TCGA and GEO cohorts. A. Cancer stage (I: open box, II: lightly shaded box, III: darkly shaded box, IV: closed box) and adipocyte density in entire TCGA and GEO cohorts. B. Pathologic grade in TCGA (G1: open box, G2: lightly shaded box, G3: darkly shaded box, G4: closed box) and GEO cohorts (G1: open box, G2: darkly shaded box, G3: Closed box) and adipocyte density. C. MKI67 gene expression by intratumoral adipocyte low (open box) vs. adipocyte high (shaded box) and Pearson correlation curve for MKI67 expression and adipocyte density in TCGA and GEO cohorts. *p*-value < 0.05 was considered statistically significant. r represents Spearman's correlation coefficient.

AL HCC had a significant increase in altered fraction, aneuploidy, and homologous recombination defects

Not only did we want to evaluate the genes active within the more aggressive AL HCC, but we also wanted to assess what underlying mutations may have occurred in AL HCC leading to these changes using scores calculated by Thorsson et al. [43]. We found that the fraction altered, the aneuploidy score, and homologous recombination defects were significantly higher in the AL HCC in the TCGA cohort (**Figure 4**). Although the number of segments appeared higher as well, this did not reach statistical significance.

Anti-cancer immune cells, CD8, Th1, and M1 cells, as well as pro-cancer Th2 cells were significantly increased in AL HCC

We also investigated the tumor immune microenvironment (TME) to assess the association of immune cells infiltraion by adipocyte infitltraion within HCC in both TCGA and GSE76427 cohorts. In the TCGA cohort, CD8 and Th1 cells were significantly increased in AL HCC (**Figure 5**). In the GSE76427 cohort, CD4, and Th1 cells were significantly increased in AL; CD8, and M1 cells also showed increased trend in AL HCC but did not reach statistical significance (**Figure 5**).

Micro-RNA miR-122 and miR-885 were significantly increased in the AL TME

Finally, we investigated the specific micro-RNA that were highly expressed in the TME of AL HCC. MiR-RNA are non-coding RNAs that epigenetically control oncogene expression and have significant role in cancer biology [45-53]. Within AL tumors, expression of miR-122 and miR-885 were significantly elevated (**Figure 6**).

Discussion

Our overarching finding was that intratumoral adipocyte-low (AL) HCC was associated with more aggressive cancer biology and worse patient prognosis and outcomes. The five-year median overall survival for HCC, in general, is approximately 19% in the current literature [54]. We found that the prognosis for AL HCC was grimmer and had significantly lower disease-free survival, disease-specific survival, and overall survival in the TCGA cohort.

To explore this, we first showed that AL HCC was associated with higher grade and had significantly increased MKI67 expression in both cohorts assessed. Higher tumor grade is known to correlate with worse overall survival in liver

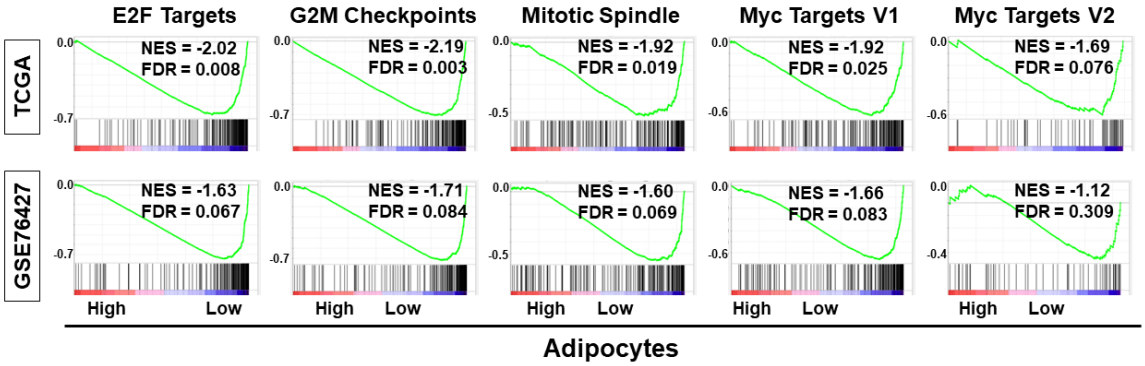

Figure 3. GSEA of adipocyte low (AL) HCC enriched Hallmark cell cycle and cell proliferation-related gene sets in the TCGA and GSE76427 cohorts. NES, normalized enrichment score; FDR, false discovery rate. FDR < 0.25 was considered statistically significant.

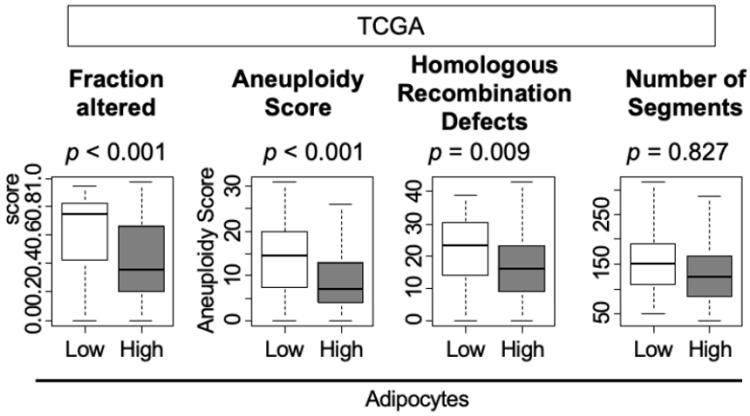

Figure 4. Fraction altered, aneuploidy score, homologous recombination defects, and the number of segments by AL and AH based on scores calculated by Thorsson et al. Intratumoral adipocyte low (open box) vs. adipocyte high (shaded box). *p*-value < 0.05 was considered statistically significant.

cancer [54, 55]. Additionally, MKI67 expression is a well-known and accepted marker for cell proliferation in cancer [15]. Specifically, in HCC, MKI67 activity has been correlated with worse tumor biology and poorer patient outcomes [56, 57]. In our study, increased MKI67 expression significantly correlated with higher adipocyte infiltration across both cohorts. However, we were unable to achieve significance when correlating adipocyte infiltration with tumor staging. A fundamental difficulty in using publicly available data is the lack of standardization and staging within the scientific community for liver cancer staging [54, 55, 58]. The TCGA and GEO cohorts used different staging systems, not allowing fair comparison between the data sets. However, AL HCC were associated with higher grade with increased MKI67 consistently in two cohorts.

To validate these pathological and molecular biological findings that cell proliferation is associated with AL HCC, we investigated what gene sets enrich to AL HCC. We found both TCGA and GSE76427 cohorts to have significant enrichment of 4 of the 5 Hallmark cell proliferation-related gene sets: E2F Targets, G2M Checkpoints, Mitotic Spindle, and MYC Targets V1. The fifth gene set, MYC targets V2, was enriched to AL in the TCGA cohort alone. E2F Targets has been identified previously as having a role in HCC proliferation, and high expression was related to poorer prognosis [59-61]. Similar findings have been associated with G2M Checkpoints, Mitotic Spindle, and MYC Targets V1 [61-65]. This validated our findings that AL HCC is associated with pathologic grade and with increased MKI67 expression, and indicates that they are highly proliferative. This underlying biology could partly explain why the tumors included in our study have low adipocyte infiltration within their tumors since the growing mass likely exerted pressure not only externally but eventually internally, and its size increased.

Recognizing that almost all cell proliferation pathways that led to the increased proliferation and cancer aggressiveness are activated in the AL HCC, we also wanted to get a sense of the genetic abnormalities and mutations that could

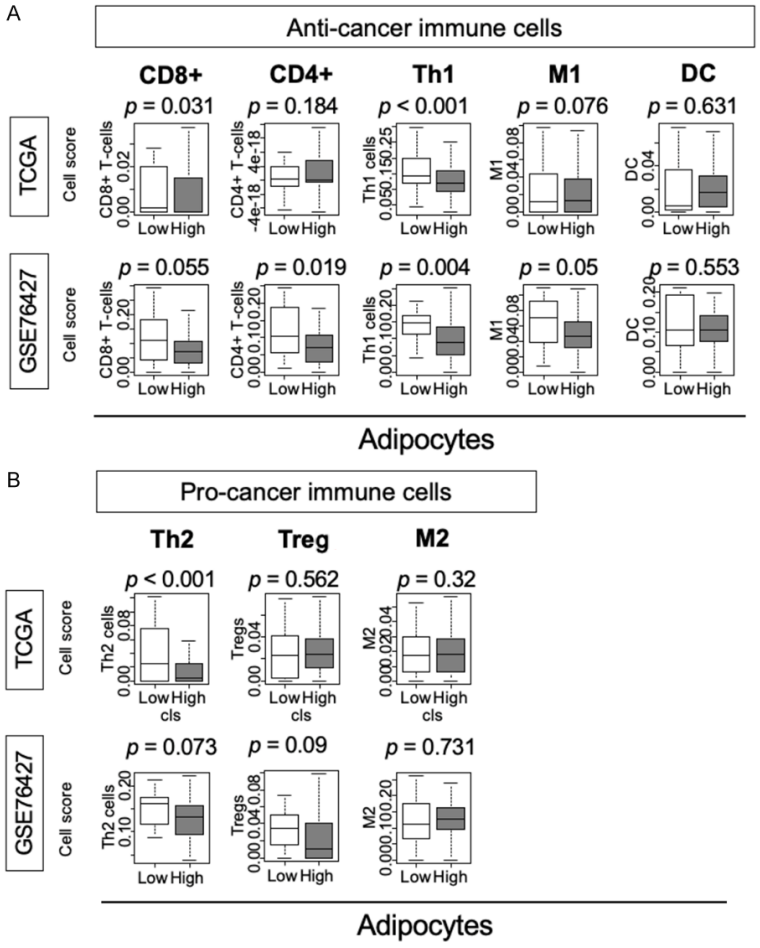

Figure 5. Immune cell composition by high and low adipocytes in the TCGA and GSE76427 cohorts. A. Anti-cancer immune cells: CD8+ (CD8+ T cell), CD4+ (CD4+ T cell), Th1 (type 1 helper T cell), M1 (M1 macrophage), DC (Dendritic cell). B. Pro-cancer immune cells: Th2 (Type 2 helper T cell), Treg (regulatory T cell), M2 (M2 macrophage). *p*-value < 0.05 was considered statistically significant.

explain the biology we had uncovered in AL tumors. We have utilized the scores calculated by Thorsson et al. [43] to see what underlying mutations could explain the gene- and subsequent phenotypic behavior of the AL HCC. It showed a significant increase in altered fraction, aneuploidy, and homologous recombination defects, significantly correlated with tumorigenesis and progression in various cancers, including HCC [43, 64, 66-68]. Again, this supported our hypothesis and conclusion that AL tumors have more aggressive cancer biology.

Additionally, although our primary interest was the role of the intratumoral adipocyte of HCC, we explored the TME to assess what anti- and pro-cancer immune cells may be infiltrated. In AL HCC, anti-cancer immune cells, CD8, Th1 (TCGA and GSE76427), and M1 (GSE76427) cells were significantly increased. In the liver, CD8 T cell upregulation has been found to correlate with liver immune pathology [69-72], but this may be due to increased reaction to the tumor as opposed to causative by the CD8 cells. Similarly, increased Th1 and M1 cells have generally been found in settings with increased anti-tumor activity or suppressed in settings of increased tumor activity [43, 72-74]. Additionally, in AL tumors, pro-cancer immune cells, Th2 cells were significantly increased (TCGA), and this trend is supported by the current literature [37, 72]. This further supports our initial hypothesis that AL HCC are more aggressive. Based upon our findings, AL HCC correlates with increased overall immune response, and both pro-and anti-cancer immune cells are significantly increased in these tumor environments.

Finally, to assess what other transcriptomic mechanisms were at play within the AL HCC, we looked at the micro-RNA expression in the AL TME. Out of the numerous miRNA assessed, only two were significantly highly expressed in AL HCC: miR-122 and miR-885. Although historical findings of miR-122 indicate that it should be decreased in more aggressive tumors since LOH of this region and subsequent down-regulation has been found in liver cancer in the past [12, 59, 75-77], we found that miR-122 was significantly elevated in the AL tumors. Conversely, other multiple studies show increased miR-122 to be associated with carcinogenesis [78]. These contradictory findings highlight an area that could benefit from further exploration. Our finding with the increased miR-885 aligns with recent studies that have noted consistently high levels in HCC patients [79, 80]. Micro-RNA is an exciting and

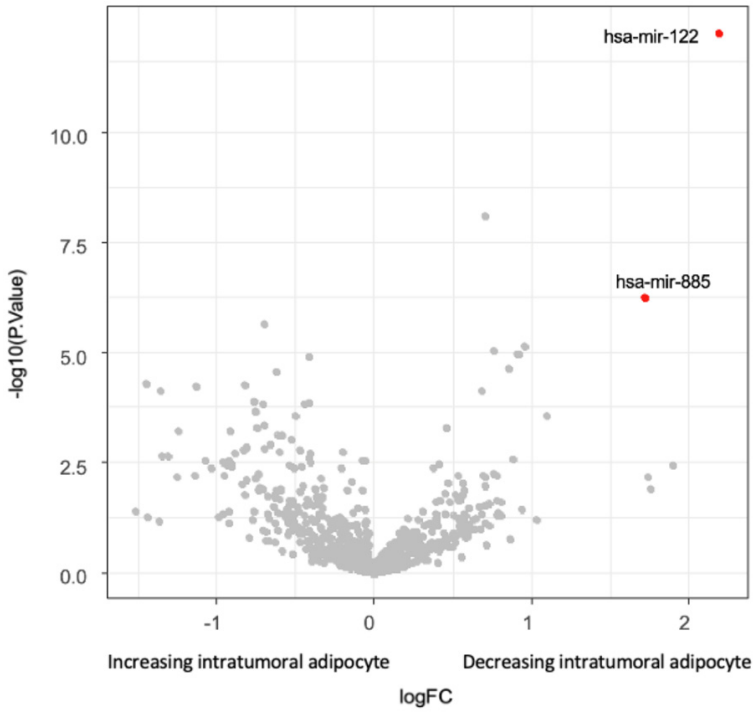

Figure 6. Volcano plot with log scale of intratumoral adipocyte density on the x-axis and -log scale of *p*-value on the y-axis. Grey dots represent miRNA frequencies that did not reach significance; orange dots represent miRNA frequencies that did reach significance. *p*-value < 0.05 and logFC > 1.5 were considered significant.

developing area of research, where there is the promise for both prognostic use and targets for intervention. We cannot help but speculate that these identified micro-RNAs might be further evaluated as diagnostic parameters, or therapeutic targets in subsequent work to improve management and patient outcomes of HCC.

Despite our best efforts, there remain some limitations to our study. We analyzed publicly available cohorts, which unfortunately did not include thorough information on the patients' complete medical history. This prevented us to determine the possible underlying cause of their HCC, whether it was infectious, environmental, due to NAFLD, or something else. Additionally, we could not use the patient's social history to correlate with smoking, diet, etc. Furthermore, although other factors were assessed similarly (and did show significant differences between AL and AH HCC), the staging of HCC was not standardized across both cohorts, so we could not compare between the two groups. A pivotal point to keep in mind with the public data used for this study is that the adipocytes assessed are specifically intratumoral and do not include peri-tumoral adipocytes, whose data was not available for analysis. This makes it difficult to draw conclusions regarding fatty liver disease, the widespread impact of hepatic adipocytes in tumorigenesis/metastasis, and the conclusions we can draw for intratumoral adipocyte low liver cancers. Therefore, extra caution is necessary when our results are compared with the other studies where adipocyte infiltration may be assessed in the peritumoral area. However, this does not change the significance of our findings and still supports the use of adipocyte density as an indicator of tumor aggressiveness and anticipated patient outcomes.

In conclusion, we confirmed our hypothesis that intratumoral adipocyte low liver cancers have more aggressive tumor biology and worse patient outcomes. Furthermore, the tumor microenvironment of AL HCC has increased infiltration of both pro and anti-cancer immune cells, and there is significant upregulation of miR-122 and miR-885 seen in other cancers compared to AH HCC. Next steps based upon our work could include assessing the relevance of adipocyte infiltration in HCC prospectively for patient outcome measures. Additionally, our work has identified vital gene sets, cell types, and microRNAs that may have a role in assessing prognosis, monitoring disease, and for possible treatment targets in hepatocellular carcinoma. Our findings help take initial steps into clarifying the role of adipocytes within HCC and provide a unique comparison to our prior work with adipocytes in breast cancer.

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Disclosure of conflict of interest

None.

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