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Original paper

NADK as a molecular marker to distinguish between alcohol- and non-alcohol-associated liver cirrhosis: A pilot study

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Abstract

Aim of the study: We investigated whether nicotinamide adenine dinucleotide kinase (NADK) expression is selectively diminished in alcohol-associated liver cirrhosis (AC), and evaluated its potential as a biomarker for this condition.

Material and methods: Human liver samples were obtained during liver transplantation or resection procedures at Kosin University Gospel Hospital and classified into two groups: AC and non-AC (NAC). NAD^+ and $NADP^+$ levels were measured using liquid chromatography-mass spectrometry (LC-MS). RNA-seq data from the NCBI Gene Expression Omnibus were utilized to identify AC-specific differentially expressed genes (DEGs). Multi-level expression analyses and immunohistochemistry were performed to assess NADK expression in liver tissues.

Results: LC-MS analysis indicated a significant reduction in NAD^+ and $NADP^+$ levels in AC patients compared to both normal and NAC groups, with a corresponding increase in the $NAD^+/NADP^+$ ratio (AC = 3.93, NAC = 2.75, normal = 2.64). We identified 881 AC-specific DEGs, including 27 kinase-encoding genes. Multi-level expression analyses confirmed a significant decrease in NADK gene expression in AC patients. Immunohistochemistry showed a marked reduction in NADK protein expression in AC patients, underscoring its involvement in altered metabolic processes.

Conclusions: This study revealed a distinct decrease in NADK expression in AC, suggesting its utility as a molecular marker for diagnosing and understanding metabolic dysregulation in these patients. These findings provide a foundation for developing targeted therapeutic strategies for alcohol-associated liver cirrhosis.

Key words: nicotinamide-adenine dinucleotide (NAD), NAD kinase (NADK), alcoholic liver cirrhosis, meta-analysis, biomarker.

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Introduction

Nicotinamide adenine dinucleotide kinase (NADK) is essential for converting NAD^+ into $NADP^+$, playing a pivotal role in cellular metabolism and antioxidant defense mechanisms [1, 2]. Deficiency of mitochondrial NADK in mouse models leads to hepatic injury, highlighting its significant role in liver metabolism [3]. In alcohol-associated liver disease (ALD), a marked reduction in the NAD^+ metabolome indicates the vital role of NADK in managing metabolic stress in liver tissues [4, 5]. Alterations in NADK2 affecting mitochondrial NADP(H) levels result in profound metabolic disturbances, emphasizing the critical role of this enzyme in maintaining mitochondrial function and overall metabolic health [6]. Collectively, these observations underscore the essential role of NADK in hepatic functions and its involvement in critical metabolic pathways.

Alcohol-associated liver disease and non-alcoholic fatty liver disease (NAFLD) are leading causes of chronic liver disease globally. While both conditions share similar histological features such as steatosis and inflammation, they differ in etiology: ALD results from excessive alcohol consumption, whereas NAFLD is associated with metabolic factors such as obesity and insulin resistance [7, 8]. Chronic alcohol intake not only causes direct hepatic injury but also leads to histological changes such as hepatocyte ballooning and fibrosis [9, 10]. The pathogenesis of alcohol-associated liver cirrhosis (AC) involves oxidative stress induced by alcohol metabolism and the toxic effects of acetaldehyde, the primary metabolic byproduct of alcohol [11, 12].

Importantly, chronic alcohol consumption induces a deficiency of niacin (vitamin B3), a crucial nutrient for NAD^+ production [13, 14], which is the essential substrate for NADK. This niacin deficiency disrupts the $NAD^+/NADP^+$ balance, critical for cellular redox homeostasis and metabolic processes, potentially impairing NADK function. The impact of alcohol on niacin levels exacerbates liver vulnerability to damage, emphasizing the need to understand how alcohol consumption affects NADK and related metabolic pathways [15].

Despite the recognized importance of NADK in hepatic functions, its relationship with alcohol consumption remains understudied. Specifically, it is unclear how alcohol-induced niacin deficiency influences NADK levels and activity in hepatic tissues. Addressing this research gap is critical for developing targeted therapeutic strategies that mitigate both the direct toxic impacts of alcohol and its indirect effect on metabolic pathways.

Our study aims to investigate the effect of alcohol consumption on NADK levels in the liver, hypothesizing that NADK is specifically decreased in AC. By elucidating this relationship, we hope to reveal novel therapeutic targets and contribute to the development of treatments for metabolic distributions in ALD, ultimately improving clinical outcomes for patients with chronic liver disease.

Material and methods

Human samples

Human liver samples were acquired at the moment of transplantation from the Kosin University Gospel Hospital. The procurement and utilization of tissue for research purposes received approval from the research ethics committee (Institutional Review Board of Kosin University Gospel Hospital, #KUGH 2024-06-019-002). All participants provided written consent for the use of their tissue samples and related data. Research activities adhered strictly to the established guidelines and legal requirements. Tissues were immediately frozen in liquid nitrogen after collection and stored at –80°C for future analysis. Specimens came from individuals without hepatocellular carcinoma or cholangiocarcinoma who were undergoing liver transplantation due to decompensated cirrhosis resulting from alcohol-associated (AC) and non-alcohol-associated (NAC) causes.

Quantitative analysis of NAD^+ and $NADP^+$

Sample processing, liquid chromatography-mass spectrometry (LC-MS), and quantification were conducted as previously detailed [16]. Given that reduced coenzymes may become oxidized and partially depleted during extraction, the measured levels of NAD^+ and $NADP^+$ serve as proxies for the total hepatic pools of NAD^+ plus NADH and $NADP^+$ plus NADPH, respectively. Typically, the majority of the combined NAD^+ and NADH pool is anticipated to be in the NAD^+ state, while the $NADP^+$ and NADPH pool is expected to predominantly exist in the NADPH state.

NCBI GEO data collection

Four datasets (GSE28619, GSE142530, GSE167308, and GSE100901) associated with AC and six datasets (GSE36411, GSE49541, GSE89377, GSE148355, GSE171294, and GSE33814) associated with NAC

were downloaded from the Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo), a public database containing high-throughput gene expression data submitted by researchers worldwide. Detailed information on these datasets is provided in Supplementary Table 1. The R package DESeq2 was used to identify DEGs from RNA-Seq data in the AC, NAC, and control groups [17]. DEGs were identified based on the criteria of |log2 (fold change)| > 1 and adjusted $p < 0.005$. The intersection function in the R package is used to find the intersection of multiple objects. DEGs between AC and normal, as well as NAC and normal, were identified and screened. DEGs specific to AC were then distinguished using Venn diagrams.

Quantitative reverse transcription polymerase chain reaction (RT-qPCR)

For the RT-qPCR analysis of patients' liver tissue, total RNA was extracted from five individual patients per group using Trizol Reagent (Invitrogen, Cat. 15596-026, Waltham, MA, USA). The same amount of RNA (1 μg) was used as a template for cDNA synthesis using oligo-dT primers and the Superscript III First-Strand kit (Cat. 18080-044, Invitrogen). Real-time qPCR was conducted using the Takara Thermal Cycler Dice Real Time System Lite (Takara Bio CO., Otsu, Japan) with 50 ng of cDNA, 4 pmoles of *NADK*-specific primers (forward: 5'-GTCCTTTGATGGACGGAAGAGAC-3', reverse: 5'- GAGGCTCTCAAACCAGTCGCTC-3') per 20 μl reaction, and TB Green Premix Ex Taq (Cat. RR420A, Takara Bio CO.) as previously described [18]. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*, forward: 5'-GTCTCCTCTGACTTCAACAGCG-3', reverse: 5'-ACCACCCTGTTGCTGTAGCCAA-3') was used as a housekeeping gene. Technical triplicates for each qPCR reaction ensured the reproducibility of the assay (independent pipetting of cDNA). The threshold cycle (Ct) value for each tissue sample and primer pair was determined, with the data representing the average from samples of five patients.

Protein extraction

Approximately 30 mg of pre-weighed liver samples (five individuals per group) were subjected to homogenization in a buffer consisting of 250 mM sucrose, 25 mM KCl, 0.5 mM EDTA, and 50 mM Tris-HCl (pH 7.4), which also contained a 1× concentration of protease and phosphatase inhibitor cocktail (Halt protease and phosphatase inhibitor cocktail, Thermo Fisher Scientific, Cat. 78441, Waltham, MA, USA). Homogenization was performed using the Fisherbrand Model 120 Sonic Dismembrator (Thermo Fisher Scientific, Cat. FB120110) at 30% pulse power, applying 10-second sonication followed by a 10-second pause for a total of 10 cycles on an ice bath. After incubating on ice for 10 minutes, the homogenate was centrifuged for 15 minutes with 13,500 g at 4°C. The supernatant was then collected, and the concentration of protein present was determined using the Bio-Rad Protein Assay Kit (Bio-Rad, Cat. 5000001, Hercules, CA, USA). The samples were then stored at –80°C until use.

Immunoblotting

After electrophoresis on a 10% SDS-polyacrylamide gel, the samples were transferred onto a polyvinylidene difluoride membrane. The membrane was blocked for 1 hour in blocking buffer (5% skim milk in Tris-buffered saline [25 mM Tris-HCl, pH 7.4, 137 mM NaCl] with 0.1% Tween 20 [TBST]) and then incubated overnight at 4°C with primary antibody solutions for NADK (1 : 1000 dilution, Proteintech, Cat. 15548-1-AP, Rosemont, IL, USA). Following this, the membrane was washed in TBST and then incubated with secondary horseradish peroxidase (HRP)-linked antibody (1 : 15000 dilution, Cell Signaling Technology, Cat. CST7074, Danvers, MA, USA) as described previously [18]. A GAPDH antibody (1 : 1000 dilution, Cell Signaling Technology, Cat. CST2118) was used to ensure uniform loading of the samples. For the detection of specific signals, an enhanced chemiluminescence method was utilized (ECL Select Western Blotting Detection Reagent, Amersham, Cat. RPN2235, Amersham, UK), following the manufacturer's protocol. Briefly, the enhanced chemiluminescence-reacted membranes were exposed to X-ray film (Medical X-ray Film Blue, AGFA, Cat.100NIF, Mortsel, Belgium) for 2 minutes, and the developed blots were scanned for band intensity quantification. The band intensity was analyzed using a luminescence image analysis system (RRID:SCR_014299, Fujifilm, Tokyo, Japan).

Immunohistochemistry and histology

Frozen human liver tissue blocks were cut into 7 μm sections and stored at –80°C. The sections were first fixed in 4% paraformaldehyde (PFA) in PBS for 10 minutes, then incubated in PBS containing 0.3% Triton X-100 (PBST). They were blocked with 5% fetal bovine serum (FBS) and 5% bovine serum albumin in PBST for 1 hour before immunohistochemistry. The primary antibody against NADK (1 : 250, Proteintech) was applied and incubated overnight at 4°C. Af-

ter washing in PBS, the sections were incubated with HRP-labeled secondary antibody (1 : 500, goat anti-rabbit, Cell Signaling Technology, Cat. CST7074) for 1 hour. Following PBS washes, sections were stained with a mixture of 3,3'-diaminobenzidine tetrahydrochloride hydrate (DAB, Sigma-Aldrich, Cat. D5637) and hydrogen peroxide (H_2O_2, Duksan, Cat. D2072, Ansan, South Korea). Slides were analyzed using a light microscope (ECLIPSE Ni Series, Nikon, Tokyo, Japan), and the intensity of the DAB staining was quantified using ImageJ software (Wayne Rasband, NIH, Bethesda, MD, USA). Staining intensity was measured under identical lighting conditions across different fields in each sample to minimize variability. Five samples were analyzed per group, and representative images were chosen to reflect the findings across all groups. For histologic analysis, sections were fixed in 4% PFA and stained with Sirius Red solution consisting of 0.1% Direct Red 80 (Sigma, Cat. 36-554-8) in saturated aqueous picric acid (Sigma, Cat. 197378) for 1 hour [19]. Quantification of collagen-stained areas (area %) in the Sirius Red-stained sections was performed using Image J software. All sections were stained and imaged under the same conditions to ensure consistency.

Statistical analysis

Statistical analyses were conducted utilizing GraphPad Prism 9 software (GraphPad Software, Inc., CA, USA). One-way ANOVA, succeeded by Bonferroni's post hoc tests for multiple comparisons, was applied when the *F*-value was significant ($p < 0.05$) and variance inhomogeneity was not significant. For comparisons between the two groups, Student's *t*-test was utilized. Additionally, Tukey's multiple comparisons tests were employed to assess significant differences among means ($p < 0.05$). The relationships between NADK expression and various clinicopathological features were assessed using Pearson's chi-square test. A *p*-value of < 0.05 was considered statistically significant for all comparisons. All data were presented as mean ± standard deviation (SD). The group size for experiments was at least $n = 5$.

Results

NAD⁺ and NADP⁺ metabolite alterations in alcohol-associated liver cirrhosis

The liver tissues were categorized into three groups: normal livers ($n = 8$), liver from AC patients ($n = 5$), and liver from NAC patients ($n = 6$). LC-MS analysis revealed differential levels of NAD⁺ among the three groups (Fig. 1A). Normal liver tissues exhibited an NAD⁺ level of 624 ±31 μM, while NAC patient tissues showed slightly lower levels at 593 ±34 μM. However, this difference was not statistically significant. In contrast, tissues from AC patients demonstrated a significant reduction in NAD⁺ level to 411 ±31 μM ($p < 0.01$ compared to normal or NAC), indicating a marked depletion of NAD⁺ in alcohol-associated cirrhosis. Further analysis assessed the levels of nicotinamide adenine dinucleotide phosphate (NADP⁺). Normal

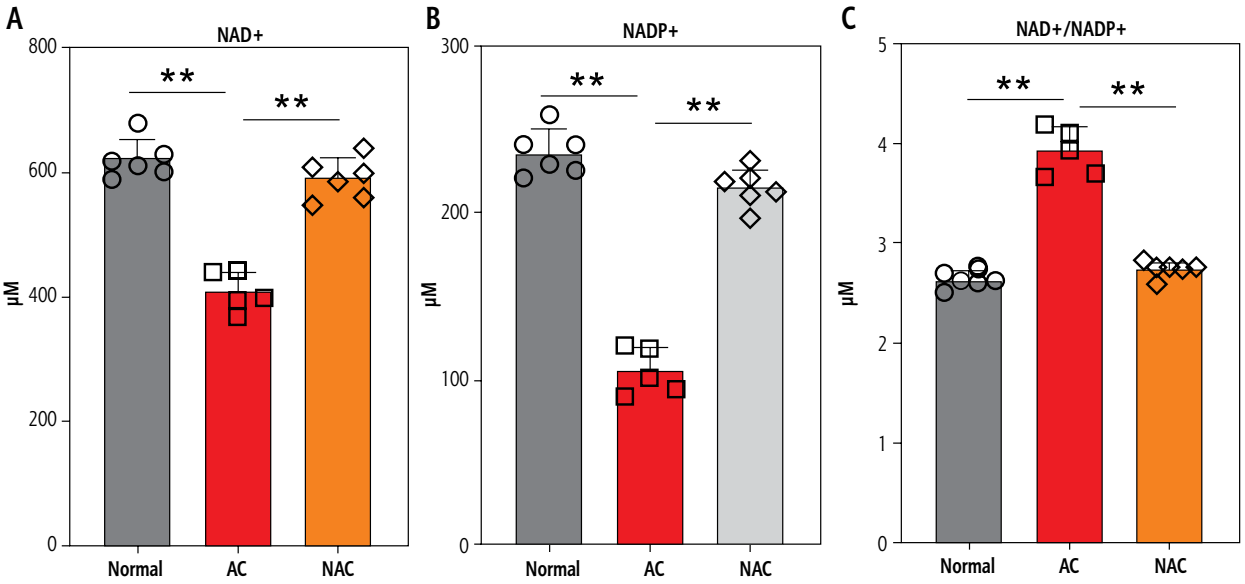

Fig. 1. Comparative analysis of NAD⁺ and NADP⁺ levels in liver tissues across different patient groups. **A**) Comparison of NAD⁺ levels across the three groups. **B**) Comparison of NADP⁺ levels. **C**) Calculated ratios of NAD⁺ to NADP⁺ for each group. Statistically significant differences are indicated, with ** denoting $p < 0.01$, compared to normal and NAC groups

liver tissues had an $NADP^+$ level of 236 ±14 μM, and NAC tissues had a level of 215 ±11 μM. Remarkably, the $NADP^+$ level in AC patient tissues was significantly lower, measured at 105 ±14 μM, indicating a more than twofold decrease compared to normal and NAC liver tissues (Fig. 1B). Consequently, the $NAD^+/NADP^+$ ratio in AC patients was elevated to 3.93, compared to 2.64 in normal tissues and 2.75 in NAC tissues (Fig. 1C). This increase in the $NAD^+/NADP^+$ ratio, alongside the decrease in both NAD^+ and $NADP^+$ levels in AC patients, highlights a distinctive metabolic alteration associated with AC. These findings suggest a profound impact of alcoholic cirrhosis on the metabolic status of liver tissues, significantly altering NAD^+ and $NADP^+$ levels and their ratio, which could have implications for understanding the biochemical pathways affected in AC.

Elucidation of AC-specific molecular alterations through RNA-seq data analysis

To elucidate the specific modifications in $NAD/NADP^+$ ratios associated with AC as shown in Figure 1, we commenced the identification of AC-specific markers by analyzing cirrhosis RNA-seq data from the NCBI GEO database. This analysis was conducted to verify the alterations unique to the molecular mechanisms of AC. A systematic approach was designed to validate distinct molecular changes linked to AC, leveraging RNA-seq data analysis for a more comprehensive understanding of the molecular framework of the disease.

Utilizing the NCBI GEO database, we meticulously identified DEGs specific to AC and NAC. For AC, DEGs that were consistently present in three or more out of four datasets examining AC were deemed AC-specific DEGs. Similarly, for NAC, DEGs found in four or more out of six datasets were classified as NAC-specific DEGs (Fig. 2A). Our analysis revealed a total of 1,240 DEGs uniquely associated with AC and 1,188 DEGs uniquely associated with NAC. Notably, there was an overlap of 359 genes between AC and NAC. In addition, we discovered 283 genes that were exclusively upregulated and 598 genes that were downregulated in AC (Fig. 2B). This comprehensive approach to analyzing RNA-seq data has not only highlighted the extent of molecular divergence between AC and NAC but also pinpointed specific genes that may underlie the distinct biochemical pathways affected in each condition. The identification of AC-specific and NAC-specific DEGs provides a foundational step toward understanding the unique molecular underpinnings of these cirrhosis subtypes.

Differential expression of kinase genes and the role of NADK in AC

Our meta-analysis identified a total of 887 DEGs specific to AC, marking a significant step toward understanding the genetic underpinnings of AC. Among these, we identified 5 genes encoding protein kinases that were up-regulated and 22 that were down-regulated (Fig. 3A).

Among the DEGs identified downregulated in our study, the *NADK* gene stood out as a pivotal discovery. NADK is critical in the metabolic conversion of NAD^+ to $NADP^+$, a key enzymatic process that is essential for maintaining cellular redox balance and overall metabolic health. The downregulation of the *NADK* gene suggests a potentially disrupted metabolic state in patients with AC, which could play a significant role in the pathology of the disease. To substantiate the findings from our comprehensive meta-analysis, we eval-

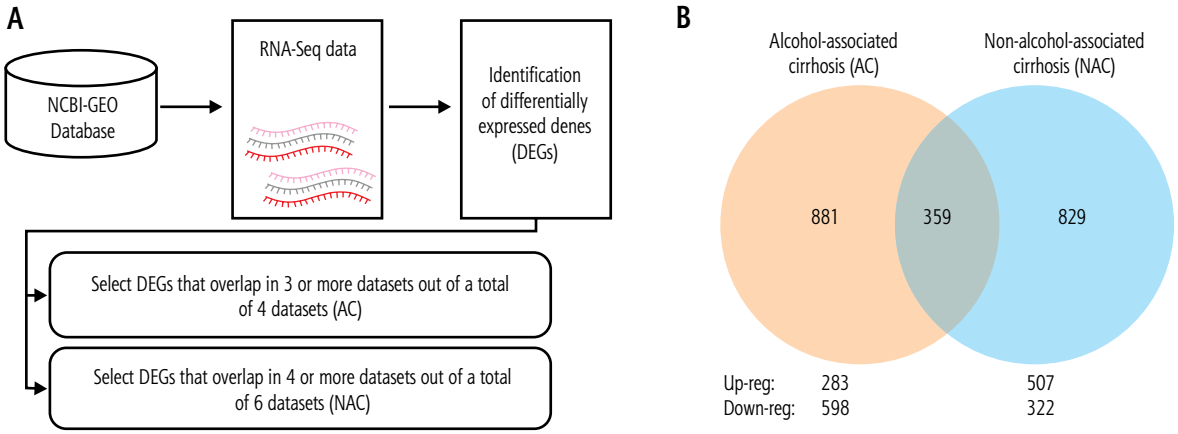

Fig. 2. Identification of alcohol-associated cirrhosis-specific targets *via* meta-analysis. **A**) A schematic diagram illustrating the analytical method employed to distinguish AC- and NAC-specific DEGs. **B**) Venn diagram summarizing the analysis of DEG lists specific to AC and NAC

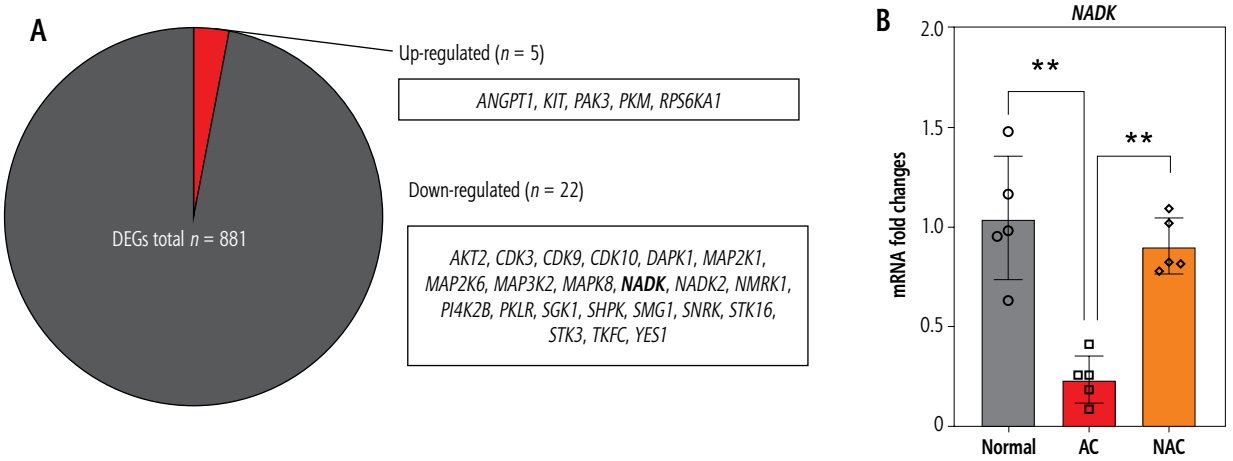

Fig. 3. Confirmation of NADK involvement and qPCR validation based on RNA-seq data. **A**) List of genes associated with 5 upregulated and 22 downregulated kinases identified among the 881 DEGs, highlighting the significant kinases including the *NADK* gene, critical for the conversion of NAD^+ to $NADP^+$. **B**) qPCR validation results for the *NADK* gene confirm its downregulation in AC patient liver samples, corroborating the RNA-seq findings. $^{**}p < 0.01$

uated the expression levels of the *NADK* gene in liver samples from patients diagnosed with AC or NAC using RT-qPCR. Our findings revealed a statistically significant decrease in *NADK* gene expression in the liver tissues of AC patients compared to those from the normal and NAC groups, as depicted in Figure 3B. This substantial reduction in *NADK* expression not only corroborates the results of our meta-analysis but also highlights the critical importance of NADK in the molecular landscape of AC. The diminished expression of NADK in liver samples from AC patients further supports its potential role in the altered metabolic processes associated with alcohol-associated cirrhosis, thereby reinforcing its relevance as a molecular marker for the disease.

Analysis of NADK protein expression in liver tissue homogenate among different cirrhosis conditions

To assess NADK protein presence in liver tissues, samples from five individuals categorized as normal, AC, and NAC were subjected to lysis for protein extraction, followed by Western blot analysis. Although five samples per group were analyzed, due to the limitations in the number of wells on the SDS-polyacrylamide gel, representative results from four samples per group are presented. GAPDH protein levels were measured as a control for normalization across the samples. Although GAPDH expression did not significantly differ among the groups, a notable decrease in NADK protein levels was observed in the AC group, as illustrated in Figure 4A. Subsequent calculations of the NADK/GAPDH ratio for each patient, and subsequent aggregation of these ratios by group, revealed a statistically significant reduction in the AC group compared to both the normal ($p < 0.03$) and NAC ($p < 0.02$) groups. No significant difference in the ratios was noted between the normal and NAC groups (Fig. 4B). This marked reduction underscores the diminished NADK protein expression in liver tissues of AC patients, highlighting a critical molecular feature of the disease.

Expression of NADK protein in liver tissues from hepatic cirrhosis patients

The expression levels of NADK protein in liver tissues were assessed through immunohistochemical analysis across three distinct groups: normal individuals, AC, and NAC. In the liver samples from normal individuals and NAC patients, robust expression of NADK protein was observed, as indicated by strong diaminobenzidine (DAB) staining. Conversely, samples from AC patients showed notably weaker staining, suggesting significantly reduced expression of NADK protein (Fig. 5A, upper panel). This visual difference underscores the diminished presence of NADK in livers affected by alcohol-mediated cirrhosis. Picrosirius red staining was used to compare the extent of fibrosis across the different groups. The results demonstrated extensive red staining in the liver tissues of both the NAC and AC groups, indicative of substantial collagen deposition, a characteristic morphological feature of cirrhosis. In contrast, normal liver tissues exhibited no apparent fibrosis (Fig. 5A, lower panel). Quantitative assessments were conducted to measure both the intensity of DAB staining and the area of collagen deposition. Analysis of DAB staining intensity, using ImageJ software on five representative photographs from each group, demonstrated statistically significant

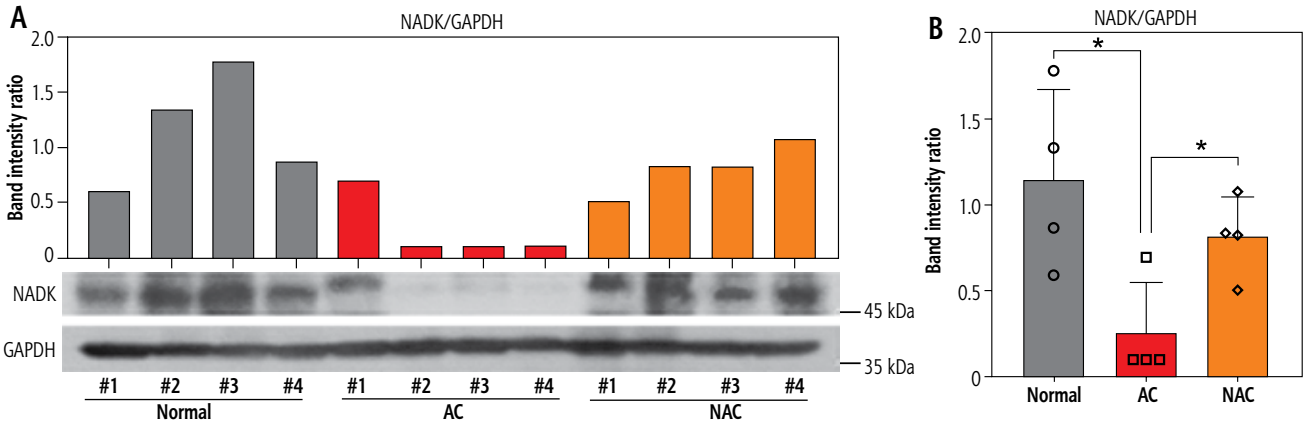

Fig. 4. Verification of NADK protein expression levels in liver tissues. **A**) Comparison of NADK and GAPDH protein expression in four representative samples from each group. **B**) Comparative analysis of the NADK/GAPDH expression ratios across the groups, quantitatively summarizing the relative abundance of NADK protein in the context of the internal control GAPDH. *$p < 0.05$

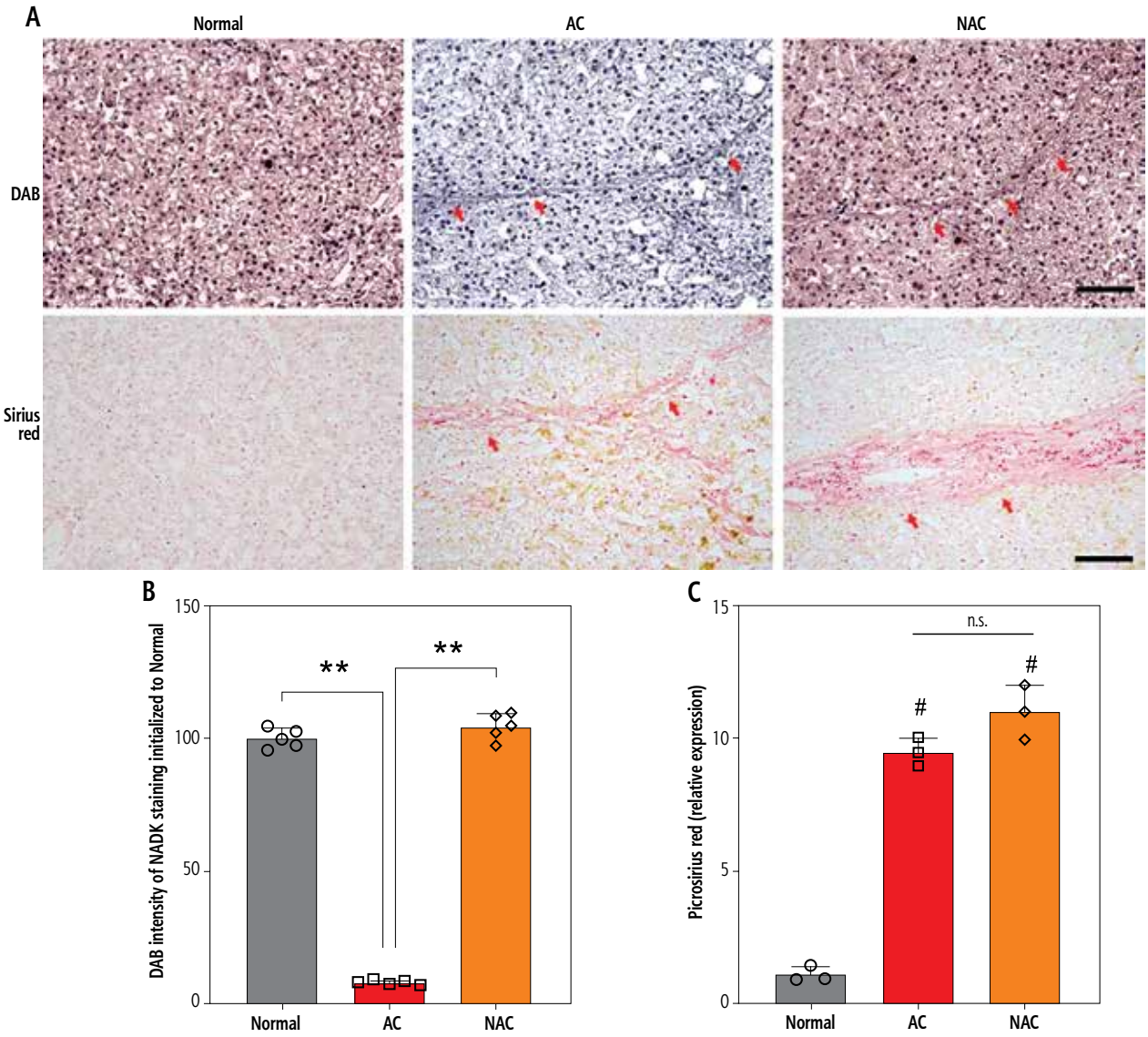

Fig. 5. Immunohistochemical and histological analyses in different patient groups. **A**) Immunohistochemical staining of NADK in normal, AC, and NAC groups. Each panel displays representative images showing the localization and expression levels of NADK (upper line). Whole liver tissues were subjected to picrosirius red staining (lower line). Red arrows indicate a fibrotic scar lesion in liver tissue. Scale bar: 100 µm. **B**) Quantification of DAB intensity for NADK expression normalized to the normal group. Data are presented as the ratio in intensity compared to the normal group, highlighting differences among the groups. **$p < 0.01$. **C**) Images were subjected to quantitative morphometry to detect Sirius red labeling, and quantitative data are depicted graphically ($n = 3$, **$p < 0.01$ compared to the normal group)

lower intensities in AC patients' liver tissues compared to normal and NAC groups (Fig. 5B, $p < 0.01$). Additionally, Figure 5C illustrates a significant increase in collagen deposition area in both AC and NAC groups, compared to normal, confirming the development of fibrosis ($p < 0.01$). These quantitative data corroborate visual assessments, confirming that while collagen deposition is observed in both AC and NAC groups, NADK protein expression is markedly decreased only in patients with alcohol-induced cirrhosis. Collectively, the findings suggest that alcohol consumption may negatively influence NADK protein expression in liver tissues, thereby potentially playing a role in the pathogenesis of alcohol-induced cirrhosis.

Discussion

Accurately distinguishing between alcohol-associated liver cirrhosis (AC) and non-AC (NAC) is crucial for the effective management and treatment of patients. Clear diagnostics not only enable the implementation of tailored therapies but also significantly enhance patient outcomes by specifically addressing the distinct pathophysiological mechanisms and complications associated with each type. For instance, treatment protocols for AC often focus on alcohol cessation alongside managing liver cirrhosis complications, while treatments for NAC, such as in cases of non-alcohol-associated fatty liver disease-related cirrhosis (NAFLD), primarily address components of metabolic syndrome [20]. Therefore, diagnostic methods must be exceptionally precise to optimize treatment efficacy and improve overall patient care, highlighting the critical need for advanced and accurate diagnostic tools. Non-invasive diagnostic techniques, such as transient elastography and other imaging methods, have been increasingly adopted to enhance the early diagnosis of liver diseases [21]. Integrating such precise diagnostic tools into clinical practice is essential for differentiating between AC and NAC effectively. This differentiation directly impacts the management strategies and therapeutic approaches, potentially reducing the morbidity and mortality associated with these liver conditions.

NAD^+ acts as a coenzyme in redox reactions, supplies ADP-ribose for ADP-ribosylation, serves as a precursor for cyclic ADP-ribose, and functions as a substrate for sirtuins (SIRTs). SIRTs utilize NAD^+ to deacetylate proteins, linking NAD^+ levels to mitochondrial functionality, dynamics, biogenesis, and cellular antioxidant defenses [22, 23]. Our findings indicate that NAD^+ levels in AC patients' livers (Fig. 1) are consistent with those reported in studies during defined periods of abstinence [4]. Notably, NAD^+ and its precursor concentrations are significantly reduced in patients with ALD and correlate with disease severity. Furthermore, an inverse relationship exists between NAD^+ levels and serum bilirubin, alongside a positive correlation with myeloperoxidase activity, suggesting that decreased NAD^+ availability may worsen impairments in antioxidant function.

In this study, we utilized RNA-seq data from the NCBI GEO database to discern the specific molecular alterations associated with AC, particularly focusing on the differential expression of genes between AC and NAC. Our analysis identified 1,240 genes uniquely altered in AC, compared to 1,188 in NAC, with a notable overlap of 359 genes, highlighting a substantial molecular divergence between these cirrhosis subtypes (Fig. 2B). Among these DEGs, kinases emerged as significant due to their regulatory roles in cellular signaling pathways [24]. Their modulatory functions in various biological processes make them ideal targets for therapeutic intervention, offering considerable advantages as disease biomarkers. The up-regulated and down-regulated kinase genes identified in AC could thus serve as noteworthy biomarkers and potential therapeutic targets.

Specifically, downregulation of the NADK gene, which encodes the only enzymes that catalyze the conversion of NAD(H) and ATP into NADP(H), was markedly observed in AC patients. This enzyme plays a crucial role in vital metabolic reactions, redox balance [1], and the maintenance of reactive oxygen species (ROS) homeostasis in cells [25]. This decrease in NADK could be linked to the impaired detoxification capacities and increased oxidative stress observed in AC patients, potentially contributing to the severity and progression of the disease. This alteration suggests a disrupted metabolic state, likely contributing to the pathology of AC. Our findings were further validated through RT-qPCR, which confirmed a significant decrease in NADK gene expression in AC patient liver samples (Fig. 3B). This evidence not only corroborates our initial RNA-seq analysis but also underscores the importance of NADK as a pivotal molecular marker in AC, suggesting its potential utility in targeted therapeutic interventions. Understanding these molecular changes provides a foundational step toward elucidating the mechanisms underlying alcohol-induced cirrhosis and offers potential targets for therapeutic intervention, underscoring the potential of genomic data in elucidating complex disease mechanisms. We also observed significant depletion of NAD^+ and $NADP^+$, and an elevated $NAD^+/NADP^+$ ratio in patients with AC (Fig. 1), indicating a severe disruption of essential metabolic pathways. This could account for the height-

ened oxidative stress and subsequent cellular damage commonly seen in scenarios of chronic alcohol exposure. This finding is consistent with Houten and colleagues [6], who noted that disturbances in mitochondrial NADP(H) levels result in significant metabolic disruptions. Thus, the diminished expression of NADK in AC may be a direct contributor to these metabolic disturbances, further aggravating the pathology of the disease.

The significant decrease in NADK protein expression (Figs. 4 and 5) identified in cases of AC underscores its role in metabolic changes linked to this condition and highlights the potential of such a marker for diagnostic purposes. Although collagen deposition was observed in both AC and NAC cases (Fig. 5A, lower panel), the differential expression of NADK suggests that patients with alcohol-associated cirrhosis may exhibit distinct metabolic characteristics. Traditional diagnostic techniques for liver cirrhosis, such as invasive biopsies and various imaging methods [26], often struggle to effectively differentiate between alcohol-associated and non-alcohol-associated forms. The discovery of biomarkers such as reduced NADK expression could lead to the development of less invasive and more precise diagnostic techniques, enabling earlier detection and facilitating more tailored therapeutic approaches. Furthermore, given the critical function of NADK in regulating cellular redox balance, strategies aimed at enhancing its activity or expression could open new therapeutic avenues. Options such as niacin supplementation or increasing levels of NAD^+ precursors might alleviate some metabolic disturbances by replenishing the substrates needed for NADK activity [27]. The ability to distinguish between AC and NAC based on NADK expression levels and their respective metabolic profiles indicates a shift towards more personalized medical approaches. Our study highlights significant differences in metabolic markers between AC and NAC. By analyzing these distinct patterns, clinicians can develop more precisely targeted interventions that address the specific underlying causes of liver cirrhosis in each patient. This tailored approach has the potential to significantly improve therapeutic outcomes by optimizing treatment strategies to better suit the individual metabolic profiles observed in different forms of cirrhosis, thus enhancing patient management and prognosis in a clinical setting.

Our study offers valuable insights but also acknowledges certain limitations that warrant attention in subsequent research. Notably, as this is a pilot study, the cohort size was relatively small, which may limit the extrapolation of our findings to broader populations. However, the insights gained from this preliminary investigation provide a valuable foundation for future, larger-scale studies. Future investigations employing larger sample sizes are essential to enhance the robustness and validate our results further. Moreover, longitudinal studies that monitor NADK levels and hepatic functionality over time would provide a more definitive basis for establishing causality and assessing the progression of AC as well as its response to therapeutic interventions. Additional research is imperative to elucidate the precise mechanisms through which NADK modulates hepatic metabolism and the specific alterations induced by alcohol consumption. Such explorations could employ experimental models that manipulate NADK expression – either through overexpression or knockdown – in hepatic cells to directly observe the consequent metabolic shifts.

In conclusion, our study demonstrates a significant reduction in NADK expression in AC, suggesting its critical role in the metabolic dysregulations associated with this condition. These findings not only enhance our understanding of the pathophysiological impacts of alcohol on the liver but also highlight the potential of NADK as a biomarker and therapeutic target in managing AC. In future, it is imperative to translate these insights into clinical applications, with the aim of enhancing diagnostic accuracy and devising targeted therapeutic strategies that specifically address the unique metabolic challenges posed by alcohol-associated liver injury.

Disclosures

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The study was approved by the Institutional Review Board of Kosin University Gospel Hospital (Approval No. #KUGH 2024-06-019-002).

The authors declare no conflict of interest.

Supplementary table is available on the journal's website.

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