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Distinct macrophage- and cardiomyocyte-associated inflammatory signatures in forensic autopsy hearts with acute ischemic heart disease

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Abstract

Sudden death due to acute ischemic heart disease (AIHD) remains a major challenge in forensic pathology, particularly in cases in which conventional macroscopic and histopathological findings are subtle or equivocal. Beyond cardiomyocyte injury, myocardial ischemia induces heterogeneous inflammatory responses arising from distinct cellular sources, including immune cells and stressed cardiomyocytes, which are difficult to evaluate using routine postmortem pathology alone. In this study, we investigated the cell-type–specific inflammatory architecture of ischemic myocardium by focusing on two inflammation-associated molecules with different biological origins: osteopontin (OPN), predominantly associated with macrophage-related immune activation, and NLRP3, reflecting cardiomyocyte-derived autoinflammatory signaling. Myocardial tissues from 78 forensic autopsy cases, including 39 AIHD cases and 39 non-AIHD controls, were examined using immunohistochemistry and double-color immunofluorescence. The AIHD group showed a significant increase in OPN-positive cells compared with controls, and OPN expression was primarily localized to TREM2-positive cardiac macrophages. In parallel, NLRP3-positive areas were significantly expanded in AIHD hearts, with NLRP3 expression predominantly observed in N-cadherin–positive cardiomyocytes. Neither OPN nor NLRP3 expression showed significant associations with age, sex, or postmortem interval. These findings demonstrate that OPN and NLRP3 reflect distinct, cell-type–specific inflammatory responses within ischemic myocardium, representing immune cell–mediated and cardiomyocyte-derived inflammatory pathways, respectively. These findings suggest that combined assessment of OPN and NLRP3 provides additional insight into the inflammatory microenvironment of ischemic myocardium and may contribute to a better understanding of postmortem myocardial pathology.

Introduction

Acute ischemic heart disease (AIHD) is characterized not only by cardiomyocyte injury but also by rapid and heterogeneous inflammatory responses that evolve within the ischemic myocardium (1, 2). These inflammatory processes arise from multiple cellular sources, including infiltrating immune cells and stressed cardiomyocytes, and contribute to tissue injury, repair, and subsequent remodeling. However, in postmortem settings, such multilayered inflammatory responses are difficult to evaluate using conventional histopathology alone, particularly in the early phase of ischemia when morphological alterations are subtle or spatially limited.

In forensic pathology, the postmortem diagnosis of AIHD remains challenging because classical macroscopic and hematoxylin–eosin–based histological findings do not always capture early or focal myocardial ischemic changes. While various immunohistochemical markers have been proposed as adjuncts to routine pathology, most have been interpreted primarily as indicators of tissue injury or cellular damage, rather than as reflections of the underlying inflammatory architecture. Consequently, the cellular origin and spatial organization of inflammation within ischemic myocardium remain insufficiently characterized in forensic autopsy material.

From this perspective, evaluating inflammatory responses according to their cellular origin may provide additional pathological context beyond conventional injury markers. Osteopontin (OPN) is predominantly associated with macrophage-related immune activation (3–9), whereas the NLRP3 inflammasome represents stress-induced autoinflammatory signaling within cardiomyocytes (10–12). Although both molecules are implicated in myocardial ischemia, their expression patterns reflect fundamentally different inflammatory pathways. Simultaneous assessment of these markers therefore offers an opportunity to visualize distinct inflammatory layers within ischemic myocardial tissue.

In the present study, we investigated the spatial and cell-type–specific expression of OPN and NLRP3 in myocardial tissue obtained from forensic autopsy cases, including AIHD and non-AIHD controls. Rather than aiming to establish diagnostic or temporal biomarkers, we sought to characterize distinct inflammatory responses associated with acute myocardial ischemia and to explore their potential value as complementary molecular pathological information in postmortem interpretation.

Materials and Methods

Human heart tissue samples

This study was a retrospective study, using existing samples and materials collected from forensic autopsies performed at the Department of Forensic Medicine, Wakayama Medical University. A total of 78 human forensic autopsy cases were included in this study (51 males; 27 females) with a PMI of less than 72 h, chosen based on autopsy documents. The individual ages ranged from 29 to 100 years (mean age, 65.5 ± 2.0). In each case, the cause of death was carefully determined through a comprehensive examination, including complete autopsy, histology, toxicology, and diatom tests. Cases were divided into two groups: 39 AIHD cases with advanced sclerosis and/or stenosis in the coronary artery and 39 others, including drowning (18 cases), fire death (6 cases), hanging (3 cases), traumatic shock (3 cases), drug and poisoning (3 cases), asphyxia (2 cases), severe head injury (2 cases), peritonitis (one case), and pneumonia (one case). The detailed profiles of all cases (sex, age, and PMI) are shown in Table 1. We selected the following study groups: AIHD, 39 cases; non-AIHD, 39 cases. The AIHD group comprised sudden cardiac death with gross and/or histopathological signs of myocardial infarction. In all cases of AIHD, the cause of death was acute myocardial infarction, Histopathological findings supportive of acute ischemic myocardial injury, including contraction band necrosis, cardiomyocyte eosinophilic degeneration/coagulative changes, early inflammatory cell infiltration, and other ischemia-associated morphological alterations, were evaluated together with macroscopic coronary findings. Cases showing putrefaction, carbonization, and traumatic injury of the heart, and cases after percutaneous coronary revascularization procedures and/or coronary artery bypass grafting were excluded. The control group (non-AIHD group), included 39 cases with no clinical history or macroscopic and microscopic findings of cardiac pathology from various causes of deaths described in Table 1.

Table 1. Forensic autopsy cases

Cause of death	n	Male/Female	Age (years)		PMI (hours)	
			Range	Mean ± SE	Range	Mean ± SE
AIHD	39	28/11	29-100	67.4 ± 2.9	3-72	26.0 ± 2.3
Non-AIHD	39	23/16	34-97	63.7 ± 2.8	10-72	29.4 ± 2.4
Drowning	18					
Fire death	6					
Hanging	3					
Traumatic shock	3					
Drug and poisoning	3					
Asphyxia	2					
Severe head injury	2					
Peritonitis	1					
Pneumonia	1					
Total	78	51/27	29-100	65.5 ± 2.0	3-72	27.7 ± 1.7

Antibodies (Abs)

Following monoclonal Abs (mAbs) and polyclonal Abs (pAbs) were used in this study: rabbit anti-human OPN pAbs (25715-1-AP, proteintech, Rosemont, IL); rabbit anti-human NLRP3 pAbs (27458-1-AP, proteintech); rabbit anti-human TREM2 pABs (bs-2723R, Bioss antibodies, Woburn, MA); rabbit anti-N-cadherin pAbs (22018-1-AP, proteintech); goat anti-mouse IgG H&L (HRP) preabsorbed (ab97040, abcam, Cambridge, UK); and goat anti-rabbit IgG H&L (HRP) preabsorbed (ab7090, abcam).

Histopathological analyses

Heart samples were fixed in 4% formaldehyde buffered with PBS and embedded in paraffin. Immunohistochemical analysis were carried out using anti-OPN pAb. Deparaffinized sections were immersed in 0.3% H_2O_2 in methanol for 30 min to eliminate endogenous peroxidase activity. The sections were further incubated with PBS containing 1% normal goat serum and 1% BSA to reduce nonspecific reactions. Subsequently, the sections were incubated with primary antibodies at 4°C overnight. After incubation with Envision+System-HRP Labelled Polymer anti-mouse (K4001, Agilent Dako, Santa Clara, CA), the immune complexes were visualized using 3,3'-Diaminobenzidine (DAB tablets, 045-22833, FUJIFILM Wako, Osaka, Japan) solution according to the manufacturer's

instructions. As a negative control, sections were incubated with normal rabbit IgG instead of primary antibodies and no positive signal was detected, indicating the specificity of the primary antibody. In addition, alkaline phosphatase (ALP) staining was carried out using anti-NLRP3 pAbs.

Morphometrical analysis

Morphometry was performed for semiquantitative evaluation as previously reported (13). For OPN, five microscopic fields (×200, 0.5×0.6 mm each) were randomly selected in each section. The number of OPN^+ cells was counted, and the total number was evaluated in each cardiac specimen. Positive reactions to NLRP3 were semiquantitatively classified as grade 0 (negative reaction), grade 1 (mild reaction), grade 2 (moderate reaction), and grade 3 (strong reaction) (14).

Double-color immunofluorescence analysis

Heart sections were analyzed double-color immunofluorescence microscopy as described previously (15). Briefly, deparaffinized sections were incubated with PBS containing 1% normal donkey serum and 1% BSA to reduce nonspecific reactions. Then, the sections were further incubated with the combination of anti-TREM2 (macrophage-associated marker) and anti-OPN, or anti-NLRP3 and anti-N-cadherin at 4°C overnight. The sections were incubated for 1 h at room temperature with the appropriate combination of following fluorochrome-conjugated secondary antibodies: goat anti-mouse IgG H&L (HRP) preabsorbed (ab97040, abcam, Cambridge, UK); goat anti-rabbit IgG H&L (HRP) preabsorbed (ab7090, abcam); and TSA Plus Fluorescence Kits (TSA Plus Fluorescein, NEL741001KT; TSA Plus Cyanine 3; NEL744001KT obtained from Akoya Biosciences Headquarters, Marlborough, MA), or Cy3-conjugated donkey anti-rat IgG pAbs (712-165-153), Cy3-conjugated donkey anti-goat IgG pAbs (705-165-147), Cy3-conjugated donkey anti-rabbit IgG pAbs (711-165-152), fluorescein (FITC)-conjugated donkey anti-goat IgG pAbs (705-095-147), FITC-conjugated donkey anti-rabbit IgG pAbs (711-095-152), and FITC-conjugated donkey anti-rat IgG pAbs (712-095-153) obtained from Jackson Immunoresearch Laboratories, West Grove, PA. Images were observed with a fluorescence microscope (BZ-X710 All-in-One Fluorescence Microscope, KEYENCE, Itasca, IL).

Statistical analysis

The AIHD and control groups were compared using a two-sided unpaired Student's *t* test to identify significant differences ($P < 0.01$ considered significant). For statistical analysis of each correlation, Pearson's product-moment correlation coefficient was used. All statistical analyses were performed using Statcel3 software under the supervision of a medical statistician as previously described (14). Normality was assessed using the Shapiro–Wilk test before statistical comparison.

Results

Enhanced OPN^+ cells in AIHD group

The number of OPN^+ cells in the heart was markedly increased in the AIHD group compared with the control group (Fig. 1A). A significant difference was observed in the number of OPN^+ cells between the AIHD group and the control group (Fig. 1B, $P = 2.87 \times 10^{-10}$). Double fluorescent staining was performed to analyze which cells expressed OPN, revealing that they were $TREM2^+$ macrophages that had infiltrated into the cardiac tissue (Fig. 2).

Correlation between OPN expression and background factors

Correlation analyses were performed to examine the relationship between the number of OPN-positive cells (per five high-power fields) and background factors including sex, age, postmortem interval (PMI), and the presence of contraction band necrosis (Fig. 3). No significant correlation was observed between OPN-positive cell counts and sex (Pearson's product–moment correlation coefficient, $r = -0.177$; Fig. 3A). Similarly, age showed no apparent correlation with OPN expression ($r = 0.156$; Fig. 3B), indicating that OPN expression was not influenced by aging. Postmortem interval also showed no meaningful association with OPN-positive cell counts ($r = -0.083$; Fig. 3C), suggesting that OPN immunoreactivity was relatively stable across the examined PMI range. In contrast, a moderate positive correlation was observed between OPN-positive cell counts and the presence of contraction band necrosis ($r = 0.452$; Fig. 3D). Cases exhibiting contraction band necrosis tended to show higher numbers of OPN-positive cells compared with cases without contraction bands, suggesting an association between OPN expression and acute ischemic myocardial changes.

Appearance of $NLRP^+$ region in hearts

When comparing the $NLRP^+$ regions between the AIHD group and the control group (Fig. 4A), the grade analysis value was clearly higher in the AIHD group (Fig. 4B, $P = 4.04 \times 10^{-14}$). To clarify which cells in the hearts of the AIHD group express NLRP3, we performed fluorescent double staining and found that they were N-cadherin-positive cardiomyocytes (Fig. 5).

Correlation analysis of myocardial NLRP3 expression

Correlation analyses were performed to evaluate the relationship between myocardial NLRP3 histological grade and demographic or pathological variables in all cases, including both non-AIHD and AIHD groups. NLRP3 expression showed no significant correlation with sex ($r = -0.156$; Fig. 6A), age ($r = 0.101$; Fig. 6B), or postmortem interval (PMI) ($r = -0.068$; Fig. 6C). In contrast, a moderate positive correlation was observed between NLRP3 expression and the presence of contraction band necrosis ($r = 0.452$; Fig. 6D), indicating that higher NLRP3 expression was associated with cases exhibiting

contraction bands. These findings suggest that myocardial NLRP3 expression is relatively independent of basic demographic factors and PMI, but is related to ischemia-associated histopathological changes.

Discussion

Postmortem evaluation of acute ischemic heart disease (AIHD) remains one of the most challenging tasks in forensic pathology, particularly in cases in which macroscopic findings and routine histopathological features are subtle, heterogeneous, or insufficient for definitive interpretation. Although conventional hematoxylin and eosin staining remains the cornerstone of pathological assessment, it does not always allow reliable characterization of early or spatially limited myocardial injury. In this context, immunohistochemical approaches should not be regarded as substitutes for routine pathology, but rather as complementary tools that may assist in visualizing underlying biological responses within myocardial tissue. The present study was therefore designed not to establish definitive diagnostic or temporal markers of myocardial ischemia, but to descriptively examine whether two inflammation-associated molecules, osteopontin (OPN) and NLRP3, exhibit distinct and cell-type-specific expression patterns in forensic autopsy cases of AIHD. By focusing on the spatial localization and cellular sources of these molecules, we aimed to provide a molecular pathological framework that may support adjunctive interpretation in selected cases where conventional morphological findings alone are limited or equivocal.

Despite ongoing efforts to improve early postmortem diagnosis of AIHD, reliable detection of very early ischemic changes remains difficult. Rather than serving as standalone diagnostic markers, additional molecular parameters that can support and refine conventional pathological evaluation may be beneficial (14, 16). Based on this concept, we examined the expression of osteopontin (OPN) and NLRP3 and assessed their possible contribution as complementary indicators in the postmortem evaluation of AIHD.

We demonstrated that OPN expression was clearly elevated in the AIHD group, and double immunofluorescence staining confirmed that the majority of OPN^+ cells were $TREM2^+$ macrophages. These observations suggest that macrophage infiltration and inflammatory activation during acute myocardial ischemia may be visualized via OPN (17–19). Furthermore, OPN expression significantly correlated with the presence or absence of contraction bands, suggesting that OPN expression reflects macrophage-associated inflammatory responses accompanying ischemic myocardial injury. OPN expression may reflect early activation of resident or rapidly recruited macrophages rather than late-stage accumulation.

On the other hand, NLRP3 is known as a component of the inflammatory inflammasome in the innate immune system, and increased NLRP3 expression was observed in the myocardial tissue of the AIHD group. Notably, NLRP3 was expressed in N-$cadherin^+$ cardiomyocytes, an important finding suggesting that cardiomyocytes represent one of the major cellular sources of NLRP3-associated inflammatory signaling in AIHD hearts. Previously, NLRP3 was reported to be primarily involved in inflammatory

responses in immune cells, but these results indicated that cardiomyocytes would autonomously activate the inflammatory cascade, potentially leading to a new understanding of the pathogenesis of myocardial ischemia (20–22). Furthermore, the association with contraction bands supports the role of OPN and NLRP3 as indicators bridging conventional histology and molecular pathology (20, 21, 23). In addition, there was a low correlation with gender, age, and PMI, demonstrating its reliability as a quantitative indicator that is less susceptible to postmortem changes.

Although both OPN and NLRP3 are involved in inflammation and tissue injury, our results suggest that they are expressed in different cell types and reflect different aspects of local inflammation (24–26). OPN may visualize macrophage-derived immune responses, while NLRP3 may visualize autoinflammatory responses within cardiomyocytes. Thus, combined evaluation of these two proteins may enable spatial and cell-specific visualization of acute inflammatory responses in AIHD.

From a practical forensic perspective, OPN and NLRP3 should not be interpreted as independent diagnostic markers, but rather as complementary molecular indicators that may provide additional contextual information when combined with established postmortem markers. Currently used immunohistochemical markers, such as fibronectin, C5b-9, troponin, and myoglobin, primarily reflect membrane injury, vascular leakage, or cardiomyocyte structural damage (14, 27–29). In contrast, OPN and NLRP3 are more closely associated with early inflammatory activation and stress-related molecular responses. Therefore, the combined assessment of conventional tissue injury markers and inflammation-related molecules may improve the overall interpretability of postmortem findings, particularly in cases with equivocal morphological changes during the very early phase of ischemia.

Based on these findings, immunohistochemical detection of OPN and NLRP3 may provide additional molecular pathological information regarding inflammatory responses associated with AIHD.

Limitations of the study

This study has several limitations that should be acknowledged. First, the present analysis was observational and descriptive in nature; therefore, causal relationships between osteopontin (OPN) or NLRP3 expression and the pathogenesis or temporal progression of acute ischemic heart disease (AIHD) cannot be inferred. Second, although the number of forensic autopsy cases analyzed was sufficient to demonstrate consistent expression patterns, the cohort size remains limited for detailed stratification according to ischemic duration or other clinical variables. Accordingly, the present findings should not be interpreted as definitive diagnostic or time-dependent markers of myocardial ischemia.

Third, immunohistochemical evaluation was performed using semiquantitative approaches, which may be influenced by sampling location and tissue heterogeneity inherent to postmortem specimens. While care was taken to standardize observation fields, more quantitative methodologies could further refine the assessment. In addition, the functional interplay between OPN- and NLRP3-associated inflammatory pathways was not directly investigated in this study. Finally, comparisons with other cardiac or systemic pathological conditions were beyond the scope of the present work and warrant future investigation.

Because forensic control cases inevitably include heterogeneous causes of death, the present findings should be interpreted as disease-associated inflammatory patterns rather than AIHD-specific diagnostic markers. Information regarding cardiopulmonary resuscitation was not consistently available in all forensic cases; therefore, contraction band necrosis was interpreted as an ischemia-associated histological finding rather than a specific marker of AIHD.

Despite these limitations, the strength of this study lies in its use of well-characterized forensic autopsy material and its focus on spatial and cell-type-specific inflammatory patterns. The present findings should therefore be regarded as providing complementary molecular pathological information that may assist postmortem interpretation in selected forensic cases, rather than as a replacement for conventional histopathological evaluation.

Declarations

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Conflict of interests

The authors declare that they have no conflict of interests.

Author contributions

Conceptualization: Y.I. and T.K., Formal analysis: Y.K., E.W., S.P., and M.N., Investigation: H.Y., M.O., and W.Z., Methodology: Y.K., Y.I., and M.N., Project administration: G.V. and T.K., Validation: R.M., M.M., and A.K., Writing-original draft: Y.I. and T.K., Writing-review & editing: G.V. and T.K..

Data availability

The authors declare that all data are available in the article file, or available from the corresponding authors upon reasonable request.

Ethical approval

This study was approved by the Research Ethics Committee of Wakayama Medical University (Approval Code. 3176; Approval Date, May 26, 2021). All the procedures were performed in accordance with the principles of the Declaration of Helsinki. Moreover, this study was conducted using past autopsy records, and informed consent was not obtained from bereaved family members for the use of these records. Therefore, we conducted this study in accordance with the "Ethical Guidelines for Medical Research Involving Human Subjects (enacted by the Ministry of Health, Labor, and Welfare in Japan), Section 12–1

(2) (a) (c).” This was a de-identified study using archived tissue obtained from judicial autopsy cases, and information on the implementation of the study was posted on our website (https://www.wakayama-med.ac.jp/dept/igakubu/160420/index.html). If they were requested to refuse the use of the samples for research, they were excluded from the study. In addition, the review board of the Research Ethics Committee of Wakayama Medical University waived the need for written informed consent from relatives of the individuals studied in accordance with national legislation and institutional requirements.

Ethical considerations: Not applicable. Anonymized case files of this retrospective

study were stored locally and data extraction was performed exclusively at the institute.

Research involving human participants and/or animal: All applicable international,

national and/or institutional guidelines and standards were followed.

Informed consent: Not applicable

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Figures

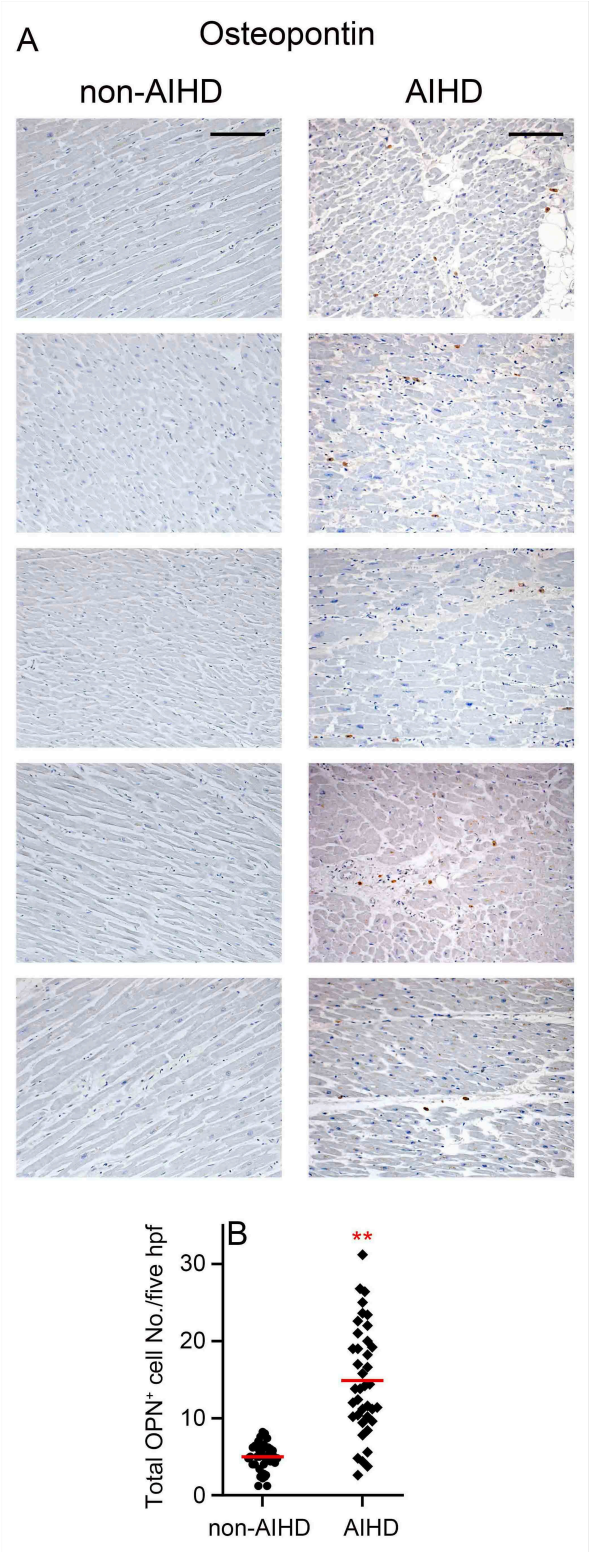


Figure 1

Expression of OPN in the human heart. (A) Immunohistochemical analysis using anti-OPN. Representative results from five hearts each from the control and AIHD groups are shown. Scale bars, 50 μm. (B) Morphometric analysis was performed to determine the number of OPN^+ cells in each group. The sum and standard error (SE) of the number of OPN^+ cells per five high power fields (hpf) for each case. $^{**}P < 0.01$ indicates a statistically significant difference.

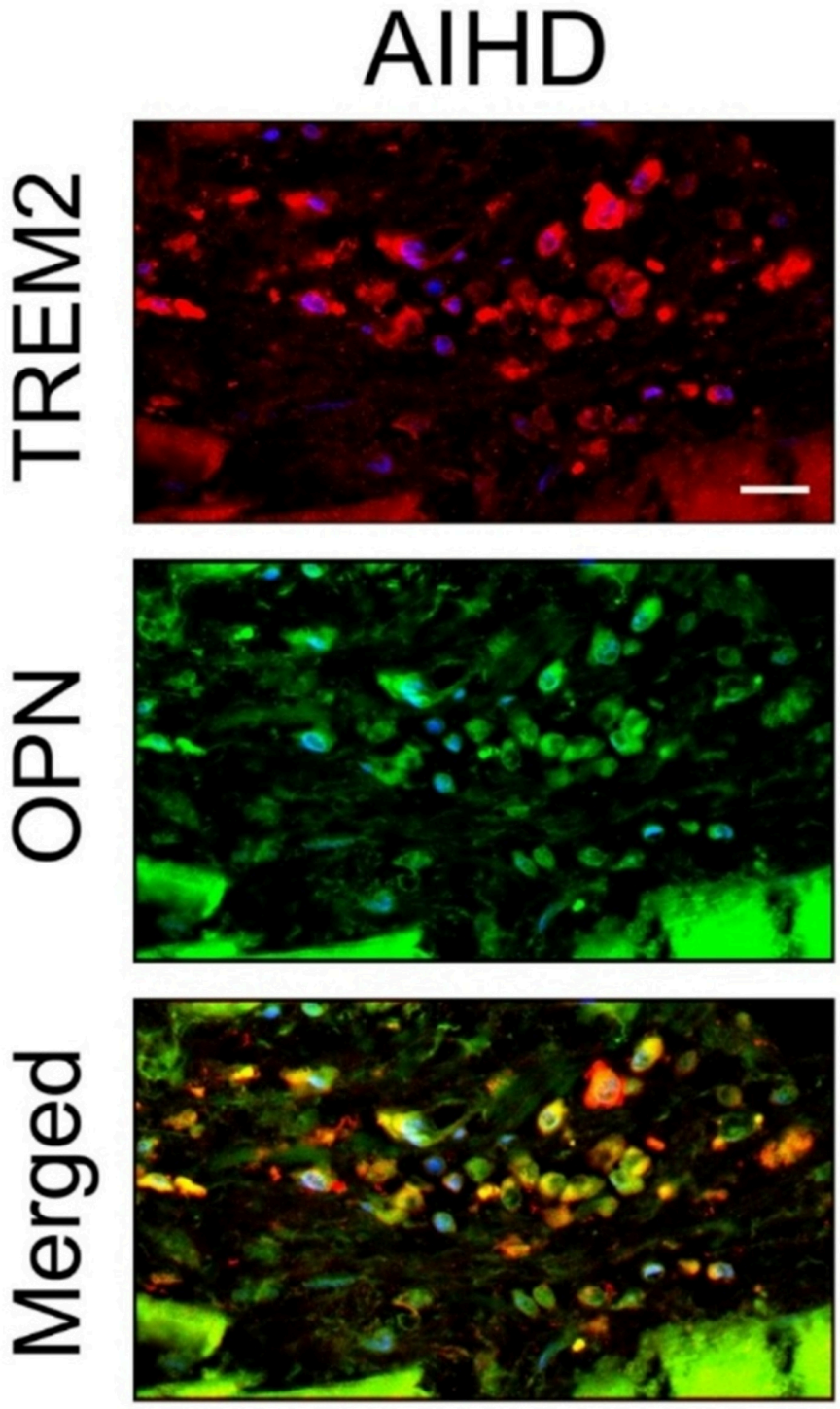


Figure 2

OPN expression in TREM2-positive cardiac macrophages in AIHD hearts. Scale bars, 20 μm.

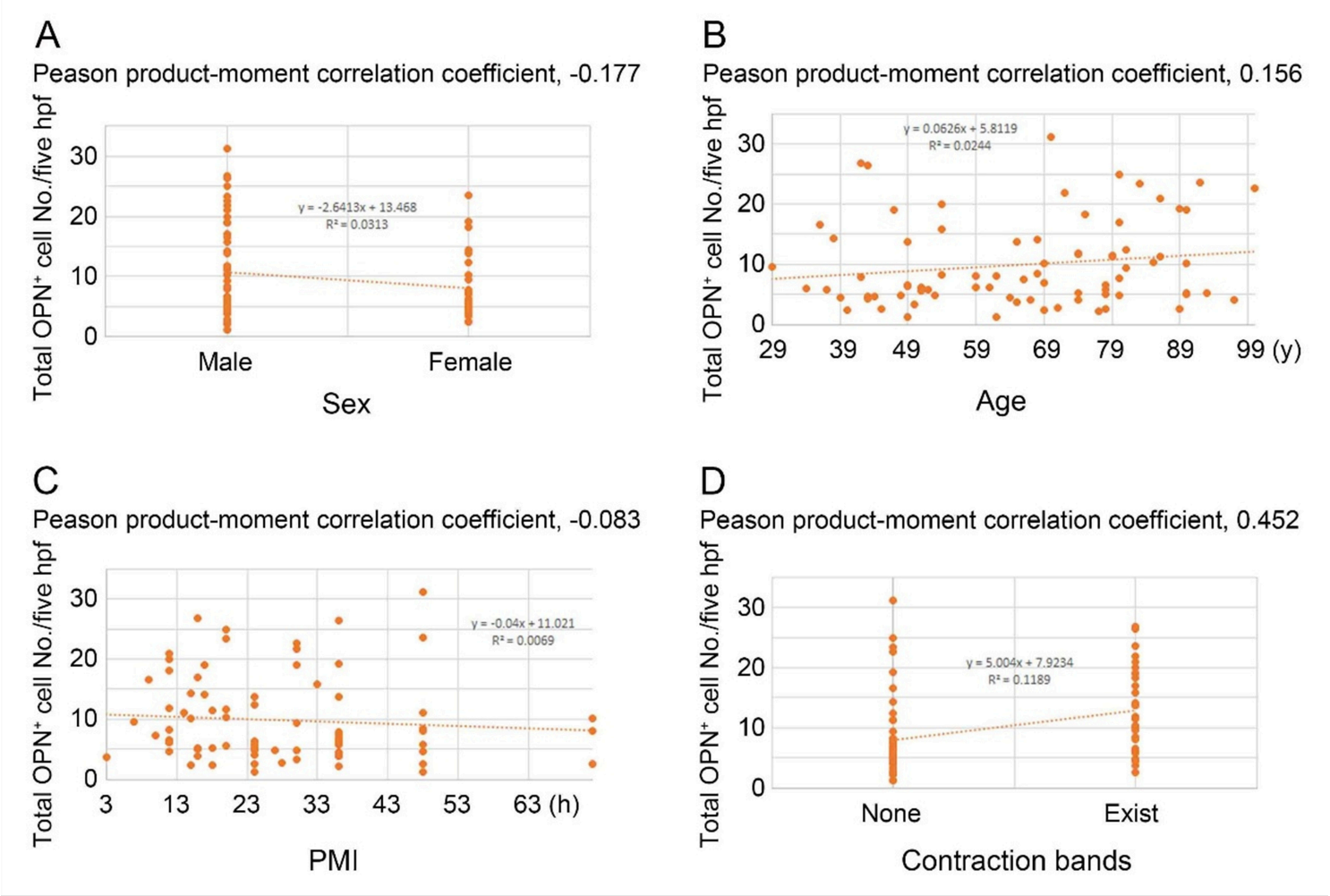


Figure 3

Correlation analysis of OPN expression in all cases (non-AIHD and AIHD cases) with sex, age, PMI, and presence or absence of contraction band. Pearson product-moment correlation coefficients are shown. (A) Sex, (B) Age, (C) PMI, (D) Presence or absence of contraction band.

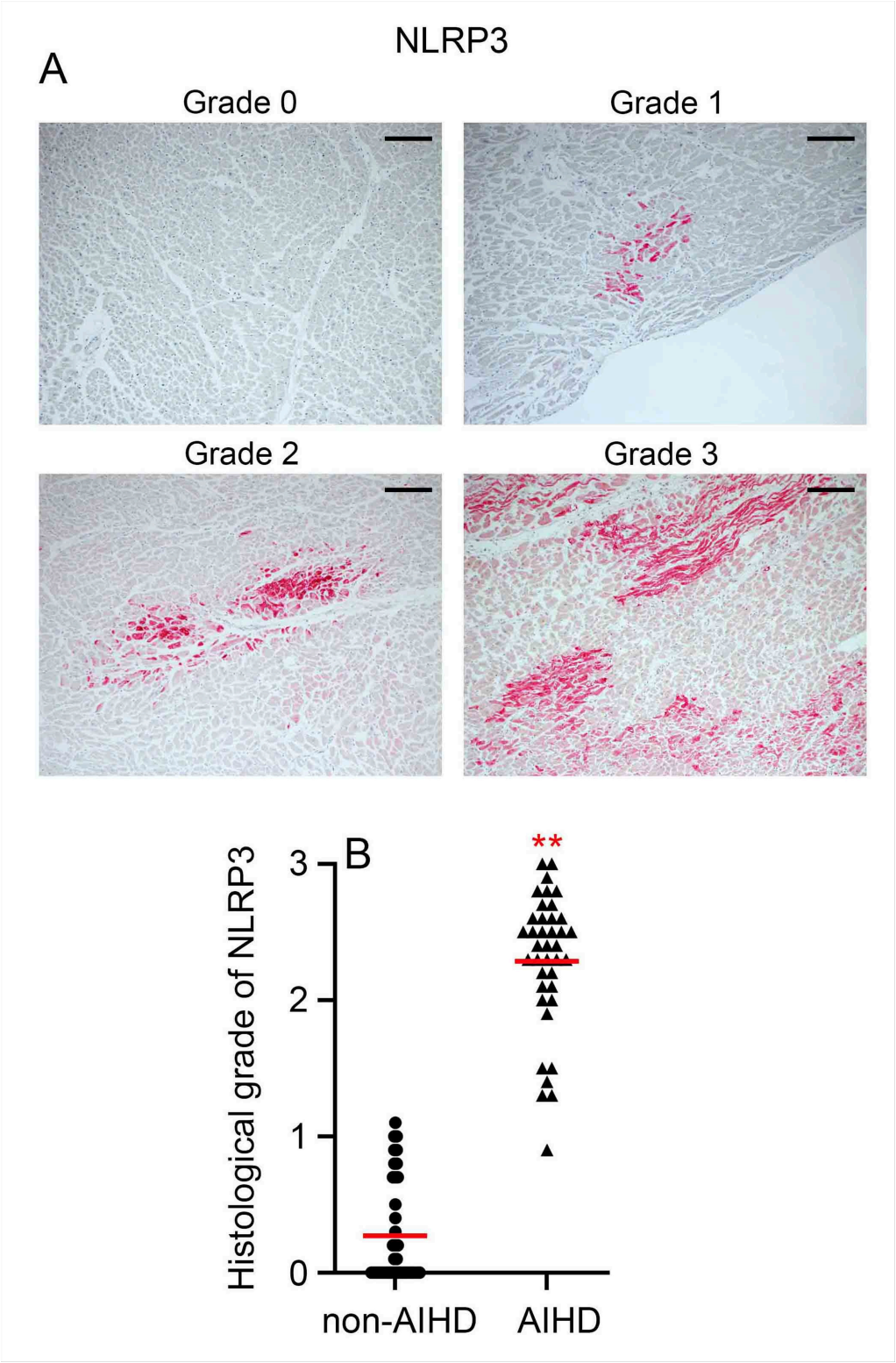


Figure 4

Expression of NLRP3 in human heart. (A) Immunohistochemical analysis using anti-NLRP3 (ALP staining). Representative results are shown, ranging from grade 0 to 3. Scale bars, 50 μm. (B) Morphometric analysis was performed to assess the grade of NLRP3 expression in each case. ***P* < 0.01 indicates a statistically significant difference.

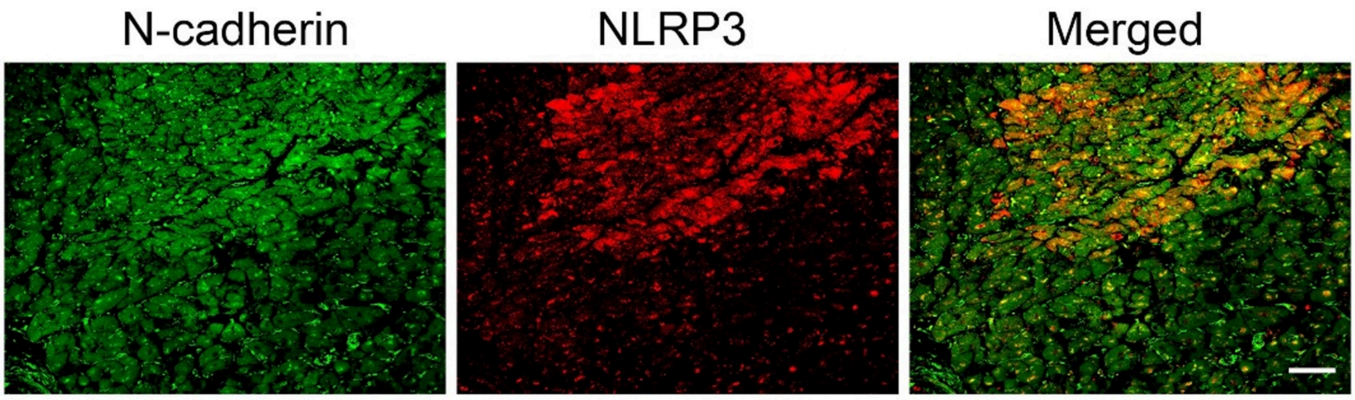


Figure 5

Figure 5

Fluorescent double staining of NLRP3 and N-cadherin protein expression. NLRP3 protein was expressed in a portion of N-cadherin-positive cardiomyocytes in AIHD hearts. Scale bar: 50 μm.

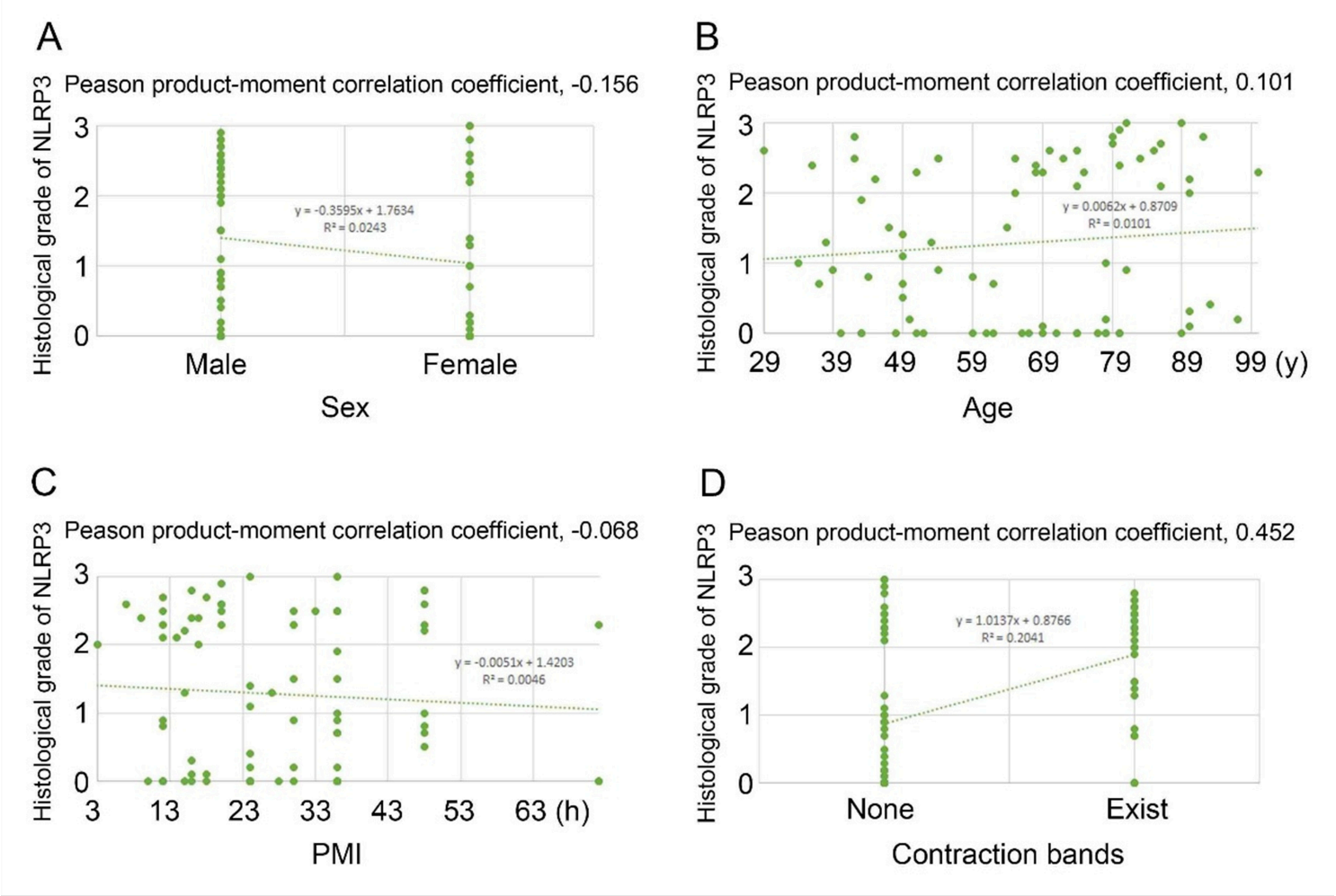


Figure 6

Correlation analysis of NLRP3 expression in all cases (non-AIHD and AIHD cases) with sex, age, PMI, and presence or absence of contraction band. Pearson product-moment correlation coefficients are shown. (A) Sex, (B) Age, (C) PMI, (D) Presence or absence of contraction band.