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Study of the expression of genes associated with post-translational changes in histones in the internal thoracic artery and the saphenous vein grafts used in coronary artery bypass grafting procedure

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Abstract

Coronary artery disease (CAD) is one of the leading causes of mortality in the world. The most advanced forms of CAD are usually treated by means of coronary artery bypass grafting (CABG). The selection of the appropriate vessels as aortocoronary conduits is of paramount importance. The internal thoracic artery (ITA) or the great saphenous vein (SV) are often harvested. Furthermore, epigenetic processes have been recently associated with atherosclerosis, hypertension, and heart failure, and post-translational histone processes may play a key role in understanding the genetic predisposition of vessels to vascular diseases.

In the experiment performed, the transcript levels of JHDM1D, PHF8, and HDAC 1-3 in SV and ITA used for CABG procedures with RT-qPCR were examined. Total RNA was isolated by the method of Chomczyński and Sachi. RNA samples were reverse transcribed into cDNA using a commercial kit. The determination of the level of the transcripts of the mentioned genes was performed using the Light Cycler® 96 Real-Time PCR kit. Our analyzes confirmed that the studied genes related to post-translational modifications of histones are expressed in SV and ITA. In the saphenous vein, the expression of each of the individual genes was higher. The most considerable difference in transcript levels was recorded for HDAC1 and the smallest difference in expression for HDAC2.

Our research suggests that more processes related to histone demethylation and acetylation occur in the saphenous vein, which may affect the selection of a vessel for CABG, but this research requires more research and additional analysis.

Running title: Histone regulating gene expression in common coronary artery bypass graft vessels

Keywords: coronary artery bypass grafting; internal thoracic artery; saphenous vein; post-translational changes in histones, JHDM1D, PHF8, HDAC1, HDAC2, HDAC3

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Introduction

Coronary artery disease (CAD) is one of the leading causes of mortality in the world. The most advanced forms of CAD are treated by means of percutaneous coronary intervention (PCI) or coronary artery bypass grafting (CABG) [1]. The latter aims to improve myocardial perfusion by implanting vascular conduits that omit obstructing atherosclerotic plaques in the coronary arteries [2–4]. The most commonly used aortocoronary conduits are the internal thoracic artery (ITA) and saphenous vein (SV). The selection of vessels for bypass grafts is of paramount importance to achieve optimal long-term outcomes, both clinical and angiographic.

SV is a superficial, large, and subcutaneous vein of the lower extremities, the transplant of which is placed in an inverted position to allow normal blood flow through the valves [4]. This vessel is ubiquitous in clinical application due to its ease of harvesting, almost unlimited length, repeatability of treatment, and quickly visible effects [1]. The most significant disadvantage of venous conduits is the suboptimal patency rate related to their premature failure leading to complete occlusion [4]. Even 20% of transplants fail only in the first year after surgery. The estimated patency rate 1, 5, and 10 years after primary surgery is respectively 78%, 65% and 57% in the case of the SV, and 93%, 90%, and 88% for ITA [5]. In daily clinical practice, the choice of vessel is not uncommonly determined by the patient's age, as SV is most often chosen in the elderly, while ITA is preferred in younger patients, usually below 60-65 years old [6].

Arterial myocardial revascularization is performed with the use of ITA, more specifically, left or right internal thoracic arteries (LITA and RITA), as well as both of those vessels (bilateral) (BITA) [6,7]. ITA provides better long-term graft patency and, consequently, survival throughout the follow-up period [4]. The advantage of ITA transplant over SV is that it is less susceptible to the development of atherosclerosis due to its own blood source, a tight, dense, and almost impenetrable internal elastic lamina, as well as a smaller number of myocytes in the adventitia [8] [9]. However, they may also be narrowed, especially in response to vasoactive drugs [10].

Many scientists have been looking for predisposing factors of preterm graft failure [11]. Among many others, they have examined metalloproteinase tissue expression [3] elevated levels of IL-6 after surgery [12], as well as tested clinical methods, both with medications (e.g. Statins) [13] or modification of surgical management [14,15] to improve the patency of a transplant after CABG. No less critical issue are the epigenetic processes, which are more and more often associated with atherosclerosis [16] and arterial hypertension [17] or heart failure [18].

Genome-wide association studies (GWAS) have identified lncRNAs that may be relevant to the genetic predisposition to cardiovascular disease (CVD), especially ischemic stroke and CAD. This concept is essential, as it explains that long non-coding RNAs are mechanistically important in CAD [19].

Epigenetics explore heritable changes in gene expression that occur without alteration of the DNA sequence [20]. Epigenetic mechanisms influence many aspects of chromatin structure or function that initiate or stop the transcription process. There are mechanisms that most often play a key role in epigenetic control: histone modification, DNA methylation, as well as patterns of microRNA expression [21,22] and ATP-dependent chromatin modifications [23]. A well-described type of post-translational modification of histones is the conservative, reversible, post-translational modification of lysine residues regulated e.g. by lysine acetyltransferases (KAT) and lysine deacetylases (KDACs; also known as histone deacetylases (HDACs). Reversible lysine acetylation plays a regulatory role in arrhythmia, heart failure, vascular disease, hypertension and angiogenesis , while HDAC inhibitors have a therapeutic role in cardiovascular disease vascular [24]. Histone deacetylase (HDACs) regulates the availability of chromatin by adding or removing the tails of histones to or lysine residues acetyl groups. HDAC remove a group of acetyl of lysine residues condensing chromatin structure and thereby inhibiting gene expression [21]. In turn, JHDM1 (JmjC domain-containing histone demethylase 1), also known as KIAA1718 or KDM7A) comprises a family of Jumonji C (JmjC) proteins and the specific demethylated histone H3 lysine 36 [25]. PHF8 has a plant homeodomain (PHD) at its N-terminus and is a member of the JmjC domain-containing protein family. PHF8 is a histone demethylase that removes the repressive traces of histone H3 dimethyl lysine 9 [26].

In the experiment performed, the levels of JHDM1D, PHF8, and HDAC 1-3 transcripts in blood vessels (SV and ITA) used for CABG procedures were examined.

Materials and methods

Operation Procedure and Sample Collection

During surgery, left ITA was used in most cases to bypass the left anterior descending coronary artery (LAD), while SV grafts were used to revascularize other target coronary arteries.

All patients were operated on from full-length median sternotomy. ITAs were harvested from the second to sixth intercostal space level as pedicled grafts, with the surrounding tissues containing satellite veins and intrathoracic fascia. After heparin administration, the ITA was cut distally, and 10 ml of papaverine solution (1 mg / ml) was carefully injected for pharmacological dilation. Before the anastomosis of the ITA graft with the recipient coronary artery, a 10 mm in length surplus distal segment of the artery was collected for laboratory tests.

In order to collect SV, the thigh skin over entire vein course was incised. The harvesting procedure based on the "non-touch" technique, including minimal manipulation of the graft, low-intensity electrocoagulation, avoiding extensive expansion, and branch control by means of stainless steel vascular clamps, was employed. In each case, about 15-20 mm of the distal part of the SV segment was preserved for further molecular analysis.

The ITA and SV vessel sets were briefly frozen in liquid nitrogen and stored at ~ 80 ° C until RNA isolation. Molecular qPCR analysis was performed on five samples: 2 SVs and 3 ITAs

RNA Extraction and Reverse Transcription

Total RNA was isolated from homogenized parts of ITA and SV samples with all vessel layers using the method developed by Chomczyński and Sachi and used in our previous studies [6,27]. RNA integrity was examined by electrophoresis using a 2% denaturing agarose gel. In the next step, the RNA concentration was determined by measuring the optical density (OD) at 260 nm (NanoDrop spectrophotometer; Thermo Scientific, Waltham, MA, USA). RNA samples were reverse transcribed into cDNA using a commercial kit kit (Roche, Germany) with the anchored-oligo (dT) 18 primer and random hexamer primer, based on the manufacturer's protocol. Samples containing 500ng RNA were used for reverse transcription.

Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR) Analysis

The experiment focused on five genes: JHDM1D, PHF8, HDAC1, HDAC2, HDAC2, HDAC3, related to post-translational changes in histones. The determination of transcripts of the mentioned gene levels was carried out using the Light Cycler® 96 Real-Time PCR kit, Roche Diagnostics GmbH (Mannheim, Germany). EvaGreen was used as the detection dye.

Each of the five samples was tested independently. Hydroxymethylbilane synthase (PBGD) and actin beta (ACTB) were used as an internal control for the analyzed transcripts. For the quantification of the target cDNA, we made a relative quantification by 2-DDCT. In order to design the primers, we used the Primer 3 software (**Tab. 1**). In order to avoid possible amplification of genomic DNA fragments, the exon-exon design method was used. The Ensembl database was also used to design primers using several transcript variants of the genes of interest. For each amplification reaction, 9 µl of the reaction mixture (Qiagen) was placed with the specified pair of primers and 1 µl of the cDNA solution was added.

Ethical approval

The research related to human use has been complied with all the relevant national regulations, institutional policies and in accordance the tenets of the Helsinki Declaration, and has been approved by the authors' institutional review board or equivalent committee. Bioethical Committee approval no. 1201/08, approved on 18/12/2008.

Informed consent statement

Informed consent has been obtained from all individuals included in this study.

Results

Our study identified the JHDM1D, PHF8, HDAC1, HDAC2, HDAC3 transcripts in both the SV and ITA segments used for coronary artery bypass surgery. RT-qPCR showed that the examined vessels ex-

TABLE 1 Oligonucleotide sequences of primers used for RT-qPCR analysis

GENE	PRIMER SEQUENCE (5'–3')
JHDM1D	**F** TCCCTTCACCTACATTTTCTG **R** TGCCTGCCTCGCCACATC
PHF8	**F** CCCTCGCCATCATTCACTG **R** TTCTCCCTCTTCCCGCTGT
HDAC1	**F** GAGACGGGATTGATGACGA **R** TGAGCCACACTGTAAGACC
HDAC2	**F** GAAGCCTCATAGAATCC **R** CTCCAACATTAAATCTCTG
HDAC3	**F** GATGACCAGACTTACAAG **R** GACATATTCAACGCATTC
PBGD	**F** GCCAAGGACCAGGACATC **R** TCAGGTACAGTTGCCCAT
ACTB	**F** GCACCACACCTTCTACAATGA **R** ATAGCACAGCCTGGATAGC

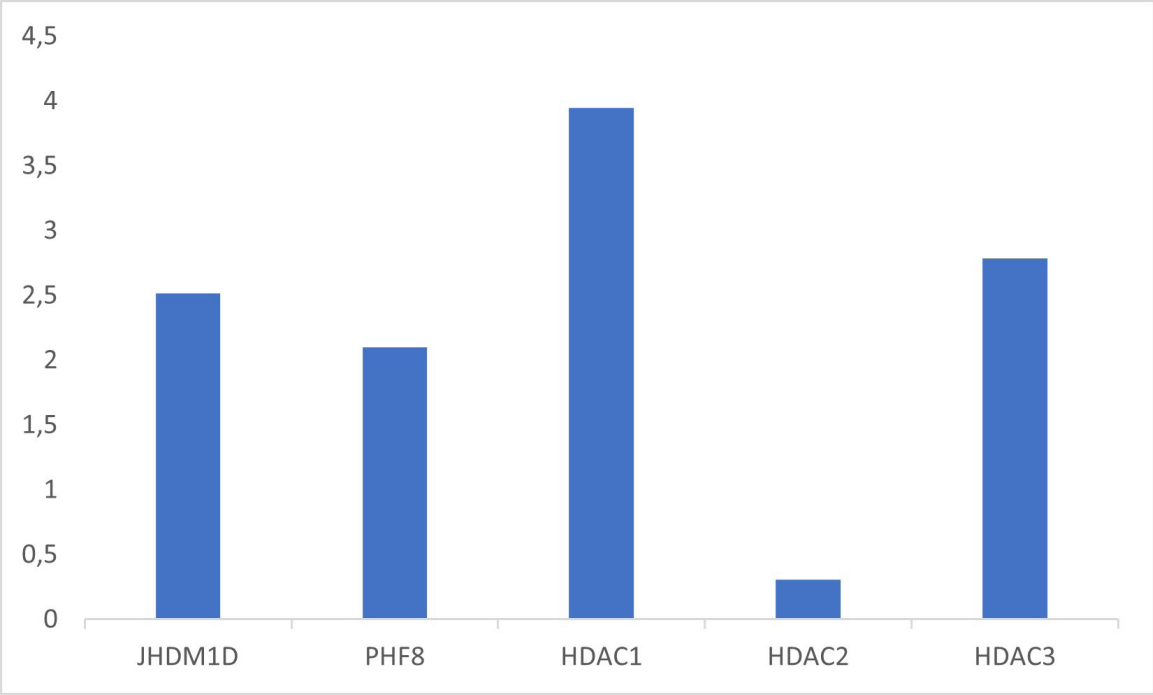


FIGURE 1 Change in the expression of genes: JHDM1D, PHF8, HDAC1, HDAC2 and HDAC3 in the saphenous vein in relation to the expression in the internal thoracic artery - vessels taken from patients who underwent CABG surgery. Total RNA was reverse transcribed and cDNA was analyzed by relative quantification by RT-qPCR. JHDM1D, PHF8, HDAC1, HDAC2 and HDAC3 mRNA levels were adjusted for PBGD and ACTB levels

pressed the genes mentioned above, while in the SV the expression of each of the individual genes was higher, as shown in **figure 1**.

The largest difference in the transcript level between SV and ITA was noted for the HDAC1 gene, while the smallest difference in expression was noted for the gene from the same family: HDAC2. The difference in gene expression for JHDM1D, PHF8, and HDAC3 was at a similar level.

Discussion

Genetic and epigenetic modifications undoubtedly contribute to the body's predisposition to the development of cardiovascular system pathologies, and research on them can improve disease prevention and treatment efficacy. Many scientists have tried to explain whether epigenetic modifications, also related to post-translational mechanisms, affect the phenotype and pathways of smooth muscle cells (SMC) in blood vessels, as well as affect the development of atherosclerosis. In particular, epigenetic processes influencing: differentiation, proliferation, and migration of SMCs, the extracellular matrix of SMC, apoptosis, vascular calcification, hypertension, diabetes, and inflammation were studied. Chromatin-related events, including DNA methylation, acetylation, and histone methylation, or SWI / SNF-mediated chromatin remodeling, regulate many different atherosclerotic processes in SMC. In particular, the differentiation, proliferation, degradation of the ECM, and SMC inflammation are defined by histone acetylation [28]. Another critical issue for the vascular system is endothelial dysfunction, which influences the development of arterial hypertension. Epigenetic processes affect endothelial function through HDAC (histone deacetylase), mediating vascular homeostasis and cardiovascular diseases[29].

This study examined the levels of JHDM1D, PHF8, HDAC1, HDAC2, and HDAC3 mRNA expression in the SV and ITA segments in patients undergoing CABG. We confirmed the presence of gene transcripts related to histone post-translational changes (JHDM1D, PHF8, HDAC1, HDAC2, and HDAC3). Differences in transcript levels in SV and ITA can affect the methyl and acetyl content.

Histones are the basic building blocks of chromatin proteins. Together with DNA, they form nucleons, i.e. the basic chromatin unit. The nucleosome core particle consists of approximately 146 bp of DNA wrapped almost twice around the histone octamer, consisting of four core histone proteins: H2A, H2B, H3, and H4 (2 copies each) [30]. Apart from their structural function, histones play an essential role in maintaining the dynamic chromatin balance that supports the regulation of gene expression. Histone tails are the amino domains protruding from nucleosomes and are susceptible to post-transposition modifications, especially the amino-terminal residues of histones H3 and H4 and the amino-terminal ends of histones H2A, H2B, and H1. Post-translational changes of histone proteins include acetyla-

tion, methylation, phosphorylation, ubiquitination, ADP ribosylation, SUMOylation, citrulline, and biotinylation [31]. Those mainly studied and described include: phosphorylation of serine and threonine residues, acetylation of lysine, methylation of lysine and arginine [31], that is, modifications of the N-terminal tails of core histones, added or removed by many specific enzymes modifying chromatin [28].

Histone methylation is a vital modification affecting both the activation and repression of transcription [21]. As mentioned above, methylation or demethylation can be performed on lysine and arginine residues in histone tails. It has been proven that the methylation of arginine 3 residues on histone H4 (H4R3) and arginine 17 on histone 3 (H3R17) may affect the activation of transcription [32]. Histone methyltransferases and lysine demethylases regulate methylation or demethylation of lysine residues on histone proteins. In contrast, acetylation or deacetylation of lysine residues on histone proteins is regulated by two opposing groups of enzymes: histone acetyltransferase (HAT) and histone deacetylase (HDAC) [30]. There are three states of lysine methylation: mono-, di- and trimethylation, which can be reversible, while the methyl groups can be removed by histone demethylases (KDM) from the LSD1 or Jumonji C-terminal (JmjC) protein family) [26].

The JmjC family of histone demethylase in humans consists of approximately 30 members [33]. The human KDM7 subfamily consists of three members: PHF2 (KDM7C), PHF8 (KDM7B), and JHDM1D (KIAA1718 / KDM7A), showing a characteristic domain organization with an N-terminal PHD domain and a JmjC catalytic domain. C-terminal regions are essential for association with RNA polymerase or transcription factors, e.g. the retinoic acid receptor (RAR), and possibly as cell cycle regulators such as E2F1, as well as contain signals for nuclear localization or phosphorylation sites [34].

Histone methyltransferases and demethylases allow the activation of the promoter (H3 4-trimethyl lysine), enhancer activity (H3K4me1), or repression (H3K9me2 / 3, H3K27me2 / 3) [35]. The catalytic role of KDM7 members in the demethylation of repressive histone markers such as H3K9me1 / 2, H3K27me1 / 2, or H4K20me1 is characterized by marked differences in substrate specificity between different enzymes [36,37]. According to Qiu et al. PHF8 has a preferential effect on H3K9me2 and H3K9me1 (possibly also being able to demethylate H4K20me1) [38]. It results in transcriptional activation and participates retinoic acid signaling pathway in neuronal differentiation. Horton et al. report that the presence of H3K4me3 on the same peptide as H3K9me2 makes the doubly methylated peptide a much better substrate for PHF8. In contrast, the opposite effect occurs when the presence of H3K4me3 reduces the activity of H3K9me2 demethylase, together with the JHDM1D substrate, without adversely affecting its H3K27me2 activity [39]. However, in the studies of Liu et al., PHF8 has been shown to function as a cell cycle regulator, based at least in part on its H4K20me1 demethylase activity. The absence of PHF8 leads to a delay in the G1 / S transition and its dissociation from chromatin in early mitosis, which, together with increased expression of Pr-Set7, leads to a sharp increase in the H4K20me1 tag capable of interacting with the Condensin II complex through HEAT repeat clusters contained in two non-SMC subunits, N-CAPD3 and N-CAPG2 [37]. PHF8 influences neuron development, and the mutations in PHF8 lead to mental retardation associated with the X chromosome [40,41]. JHDM1D, like PHF2 and PHF8, can regulate neural differentiation and development in mammals [25,42].

KDM7 members may perform pathophysiological functions and participate in the development of many diseases. Histone demethylase PHF8 has been identified as a coactivator specifically recruited by RARα fusions to activate the expression of their downstream targets after all-trans retinoic acid (ATRA) treatment in acute myeloid leukemia [43]. However, according to Xiang et al., H3K27 histone methylation may be associated with prostate cancer development and progression [44]. JHDM1D is involved in the regulation of angiogenesis, also during the development of tumors. Research by Osawa et al. shows that increased JHDM1D expression inhibited tumor growth by reducing angiogenesis under nutrient limitation [41].

However, many epigenetic mechanisms have not yet been well studied in relation to cardiovascular diseases, mainly in the vascular system. The studies by Gu et al., while investigating histone modifiers in endothelial cells, observed high expression of PHF8 and proved that PHF8 maintains endothelial function by controlling E2F4 (endothelial gene) expression. Their research noted that overexpression of PHF8 catalyzed the removal of methyl groups from histone 3, lysine 9 (H3K9), and H4K20, while enzyme knockdown increased H3K9 methylation. Knockdown of PHF8 by RNA and limited proliferation and endothelial survival, while SiRNA PHF8 impaired the ability to migrate and develop capillary-like structures [45]. Endothelial stem/progenitor cells are also of interest due to their potential use in stem cell therapy for revascularization after ischemic injury. Research indicates that epigenetic mechanisms are essential in regulating endothelial stem/ progenitor cell functions by modifying the chromatin structure [46]. On the other hand, our study provides information that JHDM1D and PHF8 gene expression levels are higher in the saphenous vein than in the internal thoracic artery. Still, for a more detailed analysis, more research is required.

As mentioned above, acetyltransferases (HAT) and histone deacetylases (HDACs) are responsible for acetylation or deacetylation of lysine residues on histone proteins, respectively, thanks to which they regulate the chromatin structure. Acetylation causes a loss of the positive charge, loosening of the chromatin structure, and allowing transcription factors to access their target genes. Histone deacetylases (HDACs) counteract histone acetyltransferases' activity by catalyzing the removal of acetyl groups from the histone tails, resulting in chromatin densification and transcription repression [46,47]. HDAC consists of 18 members grouped into four classes based on substrate, structure, sequence homology, and domain organization [28]. There are four classes of HDAC that are commonly expressed [21]. Class I HDACs, which include: HDAC1, HDAC2, HDAC3, and DAC8, are highly homologous with the yeast Rpd3 protein [47].

HDACs are strongly involved in the treatment of various diseases and cancer [48–50], as well as in vascular diseases [51–53]. Li et al. [24] report that class I HDACs protect against vascular damage but have harmful effects on the heart. Their observations have an essential impact on the clinical utility of HDAC inhibitors as therapeutic agents in cardiovascular diseases. In our study, we analyzed the transcript levels of three class I HDAC representatives: HDAC1, HDAC2, and HDAC3 of the blood vessels used for CABG. The most significant difference in the expression of the HDAC1 gene in SV compared to ITA, noted in this study, may be of significant importance for further research. Histone acetylation in vessels can be very important as demonstrated by Fish et al. [54], elevating the level of endothelial nitric oxide synthase (eNOS) mRNA in vascular smooth muscle cells (VSMC) following treatment with an HDAC inhibitor called trichostatin A (TSA). This team found that treatment with TSA depressed the endothelial line-specific eNOS gene in non-endothelial VSMCs. Therefore, it can be assumed that HDACs inhibit differentiation towards the endothelial line in cells other than EC [46]. On the other hand, Granger et al. Reported that treatment with an HDAC inhibitor reduced myocardial defect by reperfusion ischemia in mice [55]. These studies have shown that the process of acetylation/deacetylation of histones and nonhistone proteins plays an essential role in regulating EC function and differentiation.

Conclusions

Our analyzes confirmed the presence of the JHDM1D, PHF8, HDAC1, HDAC2, and HDAC3 genes in the saphenous vein and the internal thoracic artery - vessels commonly used for CABG. Furthermore, significant differences were observed in the expression of the genes of the vessels mentioned above. For each of the studied genes, SV's transcript levels were higher than in ITA, especially HDAC1. Post-translational changes of histones in blood vessels and cardiovascular diseases are of great importance. A more detailed examination of the vessels in this regard may lead to significant conclusions for the further and more successful treatment of coronary artery diseases.

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Conflict of interest statement
The authors declare they have no conflict of interest.

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