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MOLECULAR DIAGNOSTICS OF CARDIAC ALLOGRAFT REJECTION: DEVELOPMENT PATHWAYS AND FUTURE CLINICAL PROSPECTS

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Recent advances in molecular diagnostics have opened new avenues for integrating genetic and epigenetic biomarkers into clinical practice. Areas such as gene expression profiling, extracellular DNA quantification, and microRNA expression analysis have seen significant development in recent years. The diagnostic value of molecular genetic biomarkers has been demonstrated across a range of pathological conditions. Emerging clinical data now support the use of molecular diagnostics to detect post-transplant complications in recipients of solid organ transplants. In heart transplant recipients, a comprehensive assessment that includes molecular genetics, epigenetic, and clinical parameters is essential for personalized selection of immunosuppressive therapy and for prevention of graft dysfunction and vasculopathy. This review highlights the current state of molecular diagnostics in cardiac allograft rejection and explores its potential for clinical application.

Keywords: heart transplantation, gene expression profiling, extracellular DNA, miRNA, graft rejection, personalization.

INTRODUCTION

A heart transplant (HT) is generally considered the only definitive treatment option for patients with end-stage chronic heart failure that does not respond to medication. In 2023, 388 HTs were performed in the Russian Federation [1]. While advances in surgical techniques, postoperative care, and immunosuppressive protocols have significantly improved patient outcomes, the 5-year survival rate is still around 72%, and the median survival for those who survive the first year is about 13 years [2].

Acute graft rejection, both T cell-mediated rejection (TCMR) or antibody-mediated rejection (AMR), is a major hurdle to long-term survival after HT. Both types of rejection crises are directly linked to an increased risk of graft dysfunction and the development of cardiac allograft vasculopathy (CAV) [3]. To prevent TCMR, lifelong immunosuppressive therapy is required for all transplant recipients, guided by standard treatment protocols. However, a mismatch between standard drug dosages and individual patient needs can lead to adverse outcomes – insufficient dosing may result in graft rejection, while overdosing increases the risk of infections and drug toxicity.

The integration of non-invasive molecular diagnostic methods in post-transplant monitoring holds promise for enabling personalized immunosuppressive therapy, tailored to the individual characteristics of each patient. This approach may significantly reduce the incidence of post-transplant complications and prolong the heart graft function.

Posttranslational biomarkers, such as cardiac troponins T and I and brain natriuretic peptides (BNP and NT-proBNP), have shown their diagnostic value in a variety of cardiovascular diseases. Cardiac troponins, in particular, are recognized as highly sensitive and specific indicators of myocardial injury.

However, multiple studies have demonstrated that cardiac troponins lack diagnostic effectiveness in detecting acute transplant rejection in HT recipients [4]. Similarly, natriuretic peptides, including BNP and NT-proBNP, have also been found to possess insufficient sensitivity and specificity for reliable detection of post-transplant complications in this patient population [5].

In recent years, molecular genetic methods have emerged as promising tools for the diagnosis of various pathological conditions, offering the potential. Among these methods, gene expression profiling, extracellular DNA (exDNA) quantification, and microRNA (miRNA) expression analysis have shown particular diagnostic relevance.

Gene expression profiling allows identifying genes with altered expression patterns in specific disease states. Several studies have delineated distinct gene signatures associated with the development of cancers and immune-mediated disorders [6, 7]. Meanwhile, exDNA, which

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results from cellular damage and apoptosis, can be found in various body fluids including plasma, serum, urine, cerebrospinal fluid, and saliva. Elevated levels of exDNA have been reported in patients with cardiovascular conditions, including arterial hypertension, myocardial infarction, and heart failure [8].

Among the diverse group of circulating non-coding RNAs, miRNAs have gained significant attention due to their ability to regulate gene expression. Altered expression profiles of specific miRNAs have been linked to numerous diseases. Certain circulating miRNAs are elevated in the plasma of patients with coronary artery disease and acute coronary syndrome, distinguishing compared to healthy individuals [9].

The aim of this review is to examine recent advances in molecular diagnostic methods for the detection of acute transplant rejection in HT recipients, with a focus on their diagnostic effectiveness and clinical applicability.

GENE EXPRESSION PROFILING

Gene expression profiling (GEP) refers to the simultaneous measurement of the activity of a large number of genes in biological samples like blood, tissue, or cell cultures [10]. GEP of peripheral blood leukocytes can be used as a noninvasive diagnostic tool for detecting acute rejection episodes, particularly in the months following HT [11].

In a study by Horwitz et al., it was first demonstrated that GEP could be effectively used to diagnose transplant rejection in HT recipients, with results showing a strong correlation with endomyocardial biopsy findings [12]. Building on this discovery, a composite GEP test was developed, which analyzes the expression of 20 specific genes to estimate the risk of acute TCMR. This risk is quantified on a scale from 0 to 40, where a score of 34 or higher is associated with a low probability of rejection [13, 14].

This diagnostic tool is notable for its high negative predictive value (NPV >90%), making it a reliable noninvasive method to rule out acute TCMR in HT patients. However, the test has certain limitations, including a low positive predictive value (about 10%) and inability to detect acute AMR [15].

In a study by Shannon et al., a panel of nine mRNA transcripts was developed using high-throughput transcriptomic analysis to diagnose acute cellular rejection in HT recipients. A key advantage of this test is its high sensitivity in the early post-transplant period, with reliable detection as early as 55 days after transplantation [16].

GEP analysis of peripheral blood mononuclear cells and endomyocardial biopsy samples allowed us to identify gene signatures associated with AMR. These include four distinct gene sets involved in endothelial function, macrophage activity, natural killer (NK) cell activation, and interferon-γ signaling. These profiles have shown diagnostic value in identifying AMR in HT recipients [17, 18].

An emerging area of interest is the analysis of mitochondrial gene expression during transplant rejection. Tarazón et al. sequenced 112 mitochondrial-related genes in a cohort of 40 HT patients and found that expression of several mitochondrial genes was significantly elevated during episodes of acute TCMR [19]. This aligns with earlier studies suggesting that mitochondrial gene expression is upregulated during immune activation, implicating these genes not only as biomarkers but also as potential mediators of rejection [20, 21].

EXTRACELLULAR DNA

Extracellular DNA (cfDNA, cell-free DNA) is released into the bloodstream during cell apoptosis and necrosis and is a promising biomarker of organ injury [22]. In solid organ transplantation, graft injury resulting from acute TCMR or AMR leads to the release of donor-derived cell-free DNA (dd-cfDNA) into the recipient's blood [23].

Early detection methods for dd-cfDNA relied on genetic differences between donor and recipient, such as sex mismatch, human leukocyte antigen (HLA) differences, and single nucleotide polymorphisms (SNPs) [24, 25]. Later, digital droplet polymerase chain reaction (PCR) [26] and whole-genome sequencing [27] were used for more precise quantification of dd-cfDNA.

De Vlaminck I. et al. demonstrated a significant rise in dd-cfDNA levels in the blood of HT recipients during acute rejection episodes, with levels decreasing following appropriate treatment. In addition, dd-cfDNA levels increased even prior to the appearance of characteristic morphological changes in endomyocardial biopsy, indicating its potential for early detection of rejection and timely adjustment of immunosuppressive therapy [28].

A prospective multicenter study established a threshold value of 0.2% for the ratio of dd-cfDNA to total recipient cfDNA. This threshold enabled the differentiation between patients with and without acute rejection, achieving a specificity of 80%, sensitivity of 44%, and a negative predictive value of 97.1% [23].

A study by Agbor-Enoh et al. demonstrated that elevated levels of dd-cfDNA in cardiac transplant recipients correlate with the severity of both TCMR and AMR, as well as with the extent of echocardiographic changes. The authors noted that the proportion of circulating dd-cfDNA was significantly higher in patients with acute AMR compared to those with acute TCMR of the heart graft [27].

Moreover, recent findings have shown increased dd-cfDNA levels in recipients without signs of acute rejection and with verified graft vasculopathy [29].

Beyond its diagnostic value in detecting acute and chronic rejection, elevated dd-cfDNA has also been associated with the formation of donor-specific antibodies

(DSA), suggesting that subclinical graft injury may predispose to DSA formation, revealing a potential unique risk factor for sensitization [30].

MIRNAS

MicroRNAs (miRNAs, miR) are short (19–25 nucleotides), single-stranded, non-coding RNA molecules. The human genome encodes about 2,200 distinct miRs. MiRs can suppress protein synthesis by blocking translation of matrix RNA into proteins or accelerating their degradation. Because each miRNA typically controls multiple transcripts, they operate in interconnected "miR networks" that modulate entire biological pathways. Many miRs are organ- and tissue-specific, and their circulating levels are stable. These features make circulating miRNAs attractive non-invasive biomarkers for tracking post-transplant complications in solid-organ recipients [31].

Nováková T. et al. examined 11 miRNAs in biopsy samples and found that miR-144, miR-589, and miR-182 were significantly dysregulated in patients with verified acute TCMR compared with those without rejection [32].

In our previous studies, plasma levels of miR-101 and miR-27 were strong predictors of acute TCMR. Expression below the preset threshold conferred a relative risk (RR) of 1.77 ± 0.16 for miR-101 (95% CI 1.30–2.42; p = 0.0003) and RR = 1.59 ± 0.18 for miR-27 (95% CI 1.11–2.26; p = 0.011) [33].

Elevated plasma levels of miR-27 and miR-339 were associated with post-transplant myocardial fibrosis, with RR of 1.50 ± 0.16 (95% CI 1.10–2.04; p = 0.009) and 1.31 ± 0.13 (95% CI 1.02–1.69; p = 0.036), respectively [34].

Combining miRNA profiling with protein biomarkers such as ST2 and galectin-3 markedly enhanced overall diagnostic performance [35].

Analysis of 26 circulating microRNAs in HT recipients has shown that serum miR-144 levels rise in parallel with episodes of acute cellular rejection, including mild rejection graded 1R by the 2004 ISHLT criteria [36]. Diagnostic performance improves further when miR-144 is combined with miR-652, outperforming either marker alone for identifying acute cellular rejection [37].

Recent studies showing successful targeted inhibition of specific microRNAs suggest that these molecules could become therapeutic targets for slowing or preventing graft pathology in solid-organ transplant recipients.

In a porcine model of ischemia–reperfusion injury, Hinkel R. et al. demonstrated that intracoronary delivery of an antimiR-21 oligonucleotide markedly improved cardiac function while attenuating myocardial fibrosis and hypertrophy. RNA-sequencing and histological analyses confirmed lowered miR-21 expression and a reduced macrophage and fibroblast burden within the injured myocardium [38].

In a murine HT model, Lu J. et al. used an anti-miR-146a oligonucleotide to silence miR-146a. This intervention boosted autophagy in regulatory T cells, thereby strengthening their suppression of $CD4^+$ T cells and dendritic cells and, collectively, significantly ameliorated acute allograft rejection [39].

OTHER DIRECTIONS

Anti-HLA antibodies

Major histocompatibility complex molecules HLA class I (A, B and C) are expressed on all nucleated cells, while class II molecules (DPA1, DPB1, DQA1, DQB1, DRA, and DRB1) are primarily found on antigen-presenting cells, B cells, and endothelial cells. Among these, HLA-A, HLA-B, and HLA-DR are the most relevant for donor-recipient matching in organ transplantation.

Prior organ transplants, blood transfusions, implantation of circulatory assist devices, or pregnancy can lead to the formation of anti-HLA antibodies. The presence and level of these antibodies in transplant candidates are commonly assessed using panel-reactive antibody (PRA) testing. Elevated pre-transplant PRA levels are associated with an increased risk of adverse transplant outcomes [40].

The study by Sciaccaluga C. et al. evaluated the prognostic value of anti-HLA antibody detection in relation to acute graft rejection and graft vasculopathy in HT recipients. It was found that the presence of circulating anti-HLA antibodies was associated with early, mild graft dysfunction – even in the absence of verified antibody-mediated rejection or verified graft vasculopathy [41].

Donor-specific antibodies

Donor-specific antibodies (DSAs) are proteins produced by the recipient's immune system that specifically recognize and bind to donor antigens, triggering complement activation and leading to graft injury. The *de novo* formation of DSAs following heart transplantation is considered a major risk factor for the onset of antibody-mediated rejection (AMR) and is associated with poor clinical outcomes. Circulating DSAs are frequently detected in heart recipients experiencing AMR, with higher antibody titers correlating with more severe forms of rejection [42].

Moreno J.D. et al. showed elevated titers of antibodies against angiotensin II type 1 receptor (AT1R-Ab) in HT recipients with graft dysfunction. The study suggests that combining standard immunosuppressive therapy with angiotensin receptor blockers may enhance treatment efficacy in cases of AMR [43].

In the early post-transplant period, the presence of anti-endothelial cell antibodies (AECAs) has been associated with an increased risk of acute allograft rejection in cardiac recipients. Furthermore, studies have shown a correlation between the presence of antibodies targeting endothelial cytoskeletal proteins – such as vimentin,

actin, and tubulin – and a heightened risk of rejection episodes. Notably, HT recipients with diagnosed graft vasculopathy within the first five years post-transplantation exhibited significantly elevated anti-vimentin antibody titers [44].

Extracellular vesicles

Extracellular vesicles (EVs) are small (typically up to 1000 nm), spherical, membrane-bound particles released into the extracellular environment, facilitating intercellular communication under both physiological and pathological conditions – including immune activation and inflammation. Due to their presence in various biological fluids and their cargo of nucleic acids, proteins, and lipids that mirror the molecular state of their parent cells, EVs have emerged as promising noninvasive biomarkers [45].

A study by Castellani C. et al. showed a significant increase in EV levels, along with a decrease in their diameter in HT recipients with acute TCMR and AMR compared to patients without signs of rejection. The authors identified specific surface markers on EVs that were characteristic of different types of rejection. For acute TCMR, markers included CD3, CD2, ROR1, SSEA-4, HLA-I, and CD41b, while EVs associated with AMR expressed HLA-II, CD326, CD19, CD25, CD20, ROR1, SSEA-4, HLA-I, and CD41b [46].

In a study by Hu R.W. et al., donor-derived extracellular vesicles were isolated from the blood of heart recipients using antibodies targeting donor HLA class I molecules. The study found that during episodes of acute AMR, these donor EVs exhibited surface expression of the complement protein C4d – a hallmark of antibody-mediated injury. Expression of C4d on donor EVs subsided following successful treatment of the rejection episode [47].

Another study analyzed the concentration of EVs expressing tetraspanin, platelet, and endothelial markers in the plasma of HT recipients in the long-term post-transplant period (more than three years). It was found that the level of $CD90^{+}$ microvesicles was significantly higher in recipients without signs of acute rejection compared to those with biopsy-confirmed evidence of transplant rejection [48].

CONCLUSION

In recent years, numerous studies have demonstrated the effectiveness of novel molecular diagnostic approaches in verifying and predicting rejection episodes in HT recipients. These methods include the assessment of genomic, transcriptomic, and proteomic biomarkers. Implementation of such molecular diagnostics holds the potential to significantly improve long-term outcomes by enabling early detection of post-transplant complications [49].

However, despite the growing body of research in the field of noninvasive diagnostics for HT rejection, only a limited number of molecular tests have been integrated into clinical practice. This is largely due to the absence of standardized protocols and methodological limitations, such as small patient cohorts. To ensure the reproducibility, standardization, and clinical relevance of these diagnostic tools, large-scale, randomized multicenter studies are needed [50].

The study of molecular diagnostic methods for HT rejection not only enhances diagnostic accuracy and reduces reliance on invasive procedures, but also deepens our understanding of the regulatory mechanisms involved in acute TCMR and AMR. This, in turn, may pave the way for the development of novel therapeutic strategies [51].

Based on these findings, the creation of multimodal diagnostic panels appears particularly promising. These panels could integrate multiple noninvasive techniques – such as gene expression profiling, measurement of donor-derived cell-free DNA, and circulating microRNAs – to improve the detection of post-transplant complications in heart recipients [52]. Furthermore, the personalized selection of immunosuppressive therapy based on a combination of molecular-genetic, epigenetic, and clinical parameters has the potential to significantly enhance both the duration and quality of life in HT patients.

The authors declare no conflict of interest.

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