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Review:

Multifunctional protein: cardiac ankyrin repeat protein*

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Abstract: Cardiac ankyrin repeat protein (CARP) not only serves as an important component of muscle sarcomere in the cytoplasm, but also acts as a transcription co-factor in the nucleus. Previous studies have demonstrated that CARP is up-regulated in some cardiovascular disorders and muscle diseases; however, its role in these diseases remains controversial now. In this review, we will discuss the continued progress in the research related to CARP, including its discovery, structure, and the role it plays in cardiac development and heart diseases.

Key words: Cardiac ankyrin repeat protein (CARP), Cardiovascular disease, Cardiac development
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1 Introduction

Cardiac ankyrin repeat protein (CARP) was first discovered in the nucleus as a transcription co-factor to regulate cardiac gene expression in 1995 (Chu *et al.*, 1995). Subsequently, it was also found to be distributed in sarcomere I-band interacting with the giant elastic protein titin (Miller *et al.*, 2003). Since its discovery, CARP has elicited significant interest, especially in cardiovascular and muscle diseases. The expression of CARP was increased in several diseases. Moreover, CARP mutation was found in dilated cardiomyopathy and hypertrophic cardiomyopathy, which suggests its role in diagnosis and prognosis of these diseases. However, the specific role of CARP in the progress of these diseases is still inconsistent now, which implies that the discrepancy will be crucial to deepen our understanding of CARP and open new avenues for cardiovascular diseases therapy. In this study, we primarily review the structure and role of CARP in cardiac development and heart diseases.

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2 Discovery of CARP

CARP was originally identified, in 1995, as a cytokine-inducible transcription factor through screening of a complementary DNA (cDNA) library prepared from interleukin-1α and tumor necrosis factor-α-stimulated human dermal microvascular endothelial cells designated as C-193 (Chu *et al.*, 1995), and the location and sequence of C-193 and its compiled protein structure were first determined. Subsequently in 1997, rat CARP was independently isolated by three labs (Baumeister *et al.*, 1997; Jeyaseelan *et al.*, 1997; Zou *et al.*, 1997), and rabbit cDNA was then cloned and its characterization was determined in 1999 (Aihara *et al.*, 1999). In order to characterize the factors that regulate the expression of ventricular myosin light chain-2 (MLC-2v) gene, Zou *et al*. (1997) isolated the YB-1-associated nuclear factor from the neonatal rat cardiomyocyte cDNA library by performing a yeast-two-hybrid screening, and designated the results as CARP because of the repeated ankyrin protein domain and its exclusive expression in the heart. Jeyaseelan *et al*. (1997) found this protein in their search for additional cardiac-specific molecules that mediate the toxic effect of doxorubicin (DOX; adriamycin), hence they called this protein cardiac

adriamycin-responsive protein, whose expression is down-regulated in response to adriamycin. At the same time, the muscle ankyrin repeat protein (MARP) was cloned as a gene induced in the denervated skeletal muscle of adult rats, and proved to be a rodent homologue of the human's C-193 and identical to rat's CARP (Baumeister *et al.*, 1997). However, MARP was later identified as a family composed of three proteins: ankyrin repeat domain-containing protein 2 (ANKRD2), CARP, and the diabetes-related ankyrin repeat protein (DARP), which exert their functions together in the muscle (Miller *et al.*, 2003). Accumulating data showed that ANKRD2 was primarily expressed in the skeletal muscle (Singal and Iliskovic, 1998; Tsukamoto *et al.*, 2002; Miller *et al.*, 2003), while CARP was primarily expressed in the heart (Baumeister *et al.*, 1997; Jeyaseelan *et al.*, 1997; Zou *et al.*, 1997; Boengler *et al.*, 2003; Miller *et al.*, 2003) and DARP was expressed in both tissues (Ikeda *et al.*, 2003; Barash *et al.*, 2004). These represent homologous ankyrin repeat proteins, and interact together in the muscles. Recently, sheep homolog of the CARP gene was also cloned and characterized (Ma *et al.*, 2013).

3 CARP gene and protein structure

CARP is encoded by the ankyrin repeat domain 1 gene (*ankrd1*), localized in human chromosome 10q23.31 and chromosome 19C2 in mice. *Ankrd1* sequence is highly conserved among different mammalian species, with nine exons and several canonical response elements in the 5'-untranslated region, including the GATA-box, AT-rich, E-box, and TATA-box (Fig. 1). The cDNA of the human *ankrd1* is 1901 base pairs long and encodes 319 amino acids (aa) with a molecular weight of 36 kDa (Chu *et al.*, 1995; Jeyaseelan *et al.*, 1997; Zou *et al.*, 1997).

The CARP protein consists of the nuclear localization signals (NLSs) (71–80 aa, 94–103 aa), PEST-like region (108–126 aa), four ankyrin-like repeats (152–283 aa), and multiple consensus protein phosphorylation sites (Chu *et al.*, 1995; Jeyaseelan *et al.*, 1997; Zou *et al.*, 1997). The PEST-like region, enriched with proline (P), glutamic acid (E), serine (S), and threonine (T), is involved in rapid mRNA and protein degradation (Rogers *et al.*, 1986). Therefore, it has been expressed in many short-lived proteins, such as G1 cyclins (Evans *et al.*, 1983), c-myc, c-fos (Rogers *et al.*, 1986), and p53 (Gronostajski *et al.*, 1984). Ankyrin repeat protein is a 33-aa sequence motif, mediating protein-protein interactions. It has been identified in many proteins, including cyclin-dependent kinase inhibitors, cytoskeletal organizers, transcriptional regulators, and developmental regulators (Sedgwick and Smerdon, 1999). CARP also contains six calsequestrin-2 (CASQ-2)-binding sites (Torrado *et al.*, 2005) and two titin-binding sites as shown in Fig. 1.

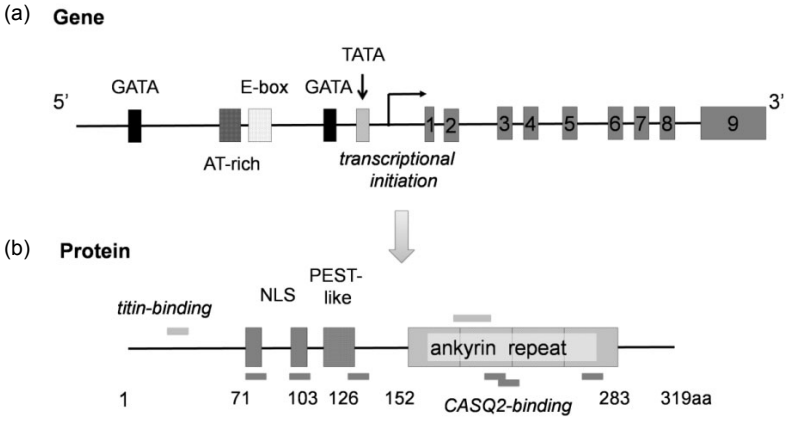

Fig. 1 Structure of the CARP gene (*ankrd1*) and translated protein

(a) *Ankrd1* sequence has 9 exons and several canonical response elements in the 5'-untranslated region, including the GATA-box, AT-rich, E-box, and TATA-box. (b) The CARP protein has 319 amino acids (aa) consisting of nuclear localization signals (NLSs) (71–80 aa, 94–103 aa), PEST-like regions (108–126 aa), and four ankyrin-like repeats (152–283 aa). CARP also contains six CASQ-2-binding sites and two titin-binding sites

4 Function of CARP

CARP has been identified in both nucleus and cytoplasm, and plays different roles in different subcellular localizations and different cell types. Currently, the role of CARP is primarily characterized in cardiac and muscle tissues.

4.1 A component of muscle sarcomere

CARP is found in the sarcomeric I-band binding to the titin-N2A element as a member of the titin mechanosensory unit (Miller *et al.*, 2003). The giant protein titin, also known as connectin, is anchored in the Z-disk and extends to the M-line region of the sarcomere (Fig. 2). It provides a structural framework through the association with other proteins of the sarcomere, keeps the thick filament centered in the sarcomere during activation, and functions as a molecular spring in the muscle sarcomere, which is involved in myocyte stress-sensing signaling (Labeit *et al.*, 1997; Granzier and Labeit, 2004). CARP plays a crucial role in maintaining sarcomeric integrity, myofibrillar signaling, and stretch sensing in the heart, interacting with other sarcomeric proteins including myopalladin (Bang *et al.*, 2001) and cardiac CASQ-2 (Miller *et al.*, 2004; Torrado *et al.*, 2005). Furthermore, CARP is also expressed in the nucleus function as a transcription co-factor; this dual localization may mediate the communication between the sarcomere and nucleus, transforming the muscle stretch signal to the gene transcription. However, the mechanisms of this process are not currently clear.

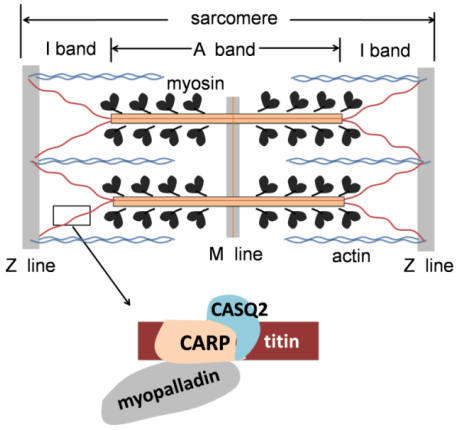

Fig. 2 CARP in sarcomere
CARP is bound to the titin-N2A elements in the sarcomeric I-band, interacting with CASQ2 and myopalladin

4.2 Nuclear transcriptional co-factor that negatively regulates cardiac gene expression and cardiac morphogenesis

CARP was initially discovered as a nuclear transcriptional co-factor, which can negatively regulate several cardiac-specific gene expressions, including MLC-2v, atrial natriuretic factor (ANF), and cardiac troponin C (cTnC) (Zou *et al.*, 1997). All of these are cardiac early genes in heart development, and CARP was discovered very early in E7.5 (7.5 d related to the presence of a vaginal plug in females, indicating that the mating occurred), suggesting its role in cardiogenesis. However, contradictory results revealed differences in the MLC-2v and cTnC transcriptions in the muscle LIM protein deficient ($MLP^{-/-}$) mice, characterized by high levels of CARP (Arber *et al.*, 1997). Therefore, further investigations are required to clarify this issue.

4.3 Enhancing neovascularization

CARP mRNA and protein were found to be dramatically up-regulated in excisional wounds (Shi *et al.*, 2005) and femoral ligation models (Boengler *et al.*, 2003), suggesting their roles in the process of angiogenesis and arteriogenesis. Additionally, CARP was also found to be expressed in the endothelial cells and smooth muscle cells (SMCs) of the collateral artery, even the inflammatory and epithelial cells within the wound. CARP overexpression could induce neovascularization and increase blood perfusion in vivo, and promote human umbilical vein endothelial cell (HUVEC) survival and migration in vitro. These mechanisms may be due to their transcriptional regulatory abilities that activate the expression of the classic angiogenic factors (vascular endothelial growth factor, hepatic growth factor, fibroblast growth factor, etc.) or inhibit the expression of angiogenic inhibitors, or paracrine effects through activating non-vascular cell types such as fibroblasts, leukocytes, or keratinocytes (Shi *et al.*, 2005). CARP may be a new target for stimulating neovascularization in ischemia tissue and wound healing. The mechanisms of CARP-induced neovascularization need to be further clarified.

5 CARP and diseases

5.1 Cardiovascular disease

CARP expression is increased in human heart failure and in different animal models of cardiac

hypertrophy. The role of CARP is primarily studied in cardiovascular and muscular diseases.

5.1.1 Cardiac hypertrophy

Cardiac hypertrophy is an adaptive response of increased heart afterload, characterized by an increase in cardiomyocyte size and enhanced cardiac fibroblast synthesis (Frey and Olson, 2003). There is now a wealth of evidence indicating that CARP expression can be markedly induced by various hypertrophic stimuli and in distinct animal models of hypertrophy, including constriction of the abdominal aorta, spontaneously in hypertensive rats and Dahl salt-sensitive rats (Aihara *et al.*, 2000a). Hypertrophic agonists activated p38 and Rac1 expression in mitogen-activated protein kinase (MAPK) pathways, which transcriptionally activate CARP expression through binding the muscle-CAT (M-CAT) elements in the promoter sequence. Of additional note, CARP can be rapidly induced and sustained in cardiac hypertrophy, which is different from other transiently increased hypertrophy-induced transcription factors (c-fos, c-jun, c-myc, and egr-1) (Chien *et al.*, 1993; Sadoshima and Izumo, 1997).

However, CARP overexpression experiments were primarily performed in vitro by transfecting the CARP gene to cardiomyocyte until Song *et al.* (2012) first generated a cardiac-specific CARP-overexpressing transgenic (CARP Tg) mice, in which they found no differences in the heart function compared with wild-type littermate. They discovered that CARP Tg mice developed less hypertrophy than wild-type mice in cardiac hypertrophy models, including transverse aortic constriction (TAC) and isoproterenol, and concluded that CARP could attenuate cardiac hypertrophy mediated by inhibition of extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) and transforming growth factor β (TGF-β) pathways and then decrease fibrosis deposition in the heart. Conversely, Bang *et al.* (2014) found that CARP is not essential for normal cardiac development and functions in basal conditions and in response to mechanical pressure overload; Chen *et al.* (2014) demonstrated that CARP promoted cardiomyocyte hypertrophy through calcineurin accumulation.

By analyzing 384 hypertrophic cardiomyopathy (HCM) patients, Arimura *et al.* (2009) detected three *ankrd1* missense mutations (Pro52Ala, Thr123Met, Ile280Val) in HCM patients, and all mutations showed increased binding of CARP to both titin and myopalladin. These findings suggest that the binding of sarcomeric CARP to titin and myopalladin plays a pivotal role in maintaining the cardiac function. Subsequently, Crocini *et al.* (2013) investigated the effects of HCM-associated mutations on contraction ability after gene transfer in engineered heart tissues, providing evidence that CARP mutations influenced myocyte contractions through different mechanisms.

5.1.2 Dilated cardiomyopathy

Ankrd1 was identified as a new gene associated with dilated cardiomyopathy (DCM) by two labs independently (Duboscq-Bidot *et al.*, 2009; Moulik *et al.*, 2009). Genetic and functional analyses in cardiomyocytes demonstrated that *ankrd1* mutations can impair CARP's nuclear function. Similarly, three missense heterozygous *ankrd1* mutations (P105S, V107L, M184I) were discovered in 4 DCM patients after screening for mutations of *ankrd1* in 208 DCM patients (Moulik *et al.*, 2009). While performing in vitro functional assays, Moulik *et al.* (2009) found that the CARP mutations altered CARP-associated protein interactions and expressions of proteins involved in key cellular pathways, such as cell cycle (p53, myogenin), apotosis (p53), growth (TGF-β, early growth response protein 1), and calcium signaling proteins (troponin T, CASQ2) when compared with wild-type CARP. These mutations resulted in disruption of the normal cardiac stretch-based signaling, providing a new pathway associated with DCM in addition to the abnormalities in structural components of the sarcomere and cytoskeleton. However, because their experiments were performed using in vitro models, these findings still need to be evaluated in animal models.

5.1.3 Adriamycin (doxorubicin)-induced cardiomyopathy

Adriamycin (DOX) is an effective chemotherapeutic agent used frequently to treat many human neoplasms, including breast cancer, leukemia, and sarcomas (Bristow *et al.*, 1978). However, severe cardiotoxicity of DOX limits its clinical use (Steinherz *et al.*, 1991; Singal and Iliskovic, 1998). The proposed mechanism for DOX-induced cardiomyopathy is the production of reactive oxygen species in cardiomyocyte mitochondria (Doroshow *et al.*, 1980;

Yen *et al.*, 1996; Zhou *et al.*, 2001). The characteristic features of DOX-induced cardiomyopathy are the loss of myofibrils and the vacuolization of cardiac myocytes (Singal and Iliskovic, 1998).

Previous studies demonstrated that DOX also depleted GATA4 expression in cardiomyocytes, and preservation of GATA4 levels prevented DOX-induced cardiomyocyte death (Kim *et al.*, 2003; Aries *et al.*, 2004). CARP was once called the cardiac adriamycin-responsive protein, because of the fact that its expression is down-regulated in response to adriamycin (Jeyaseelan *et al.*, 1997), which was confirmed in subsequent studies (Aihara *et al.*, 2000b). Of note, CARP was reported as a downstream target of GATA4 (Kuo *et al.*, 1999; Kim *et al.*, 2003; Chen *et al.*, 2012). Therefore, GATA4 and CARP would be therapeutic targets in DOX-induced cardiomyopathy.

5.1.4 Cardiac ischemia injury and myocardial apoptosis

Hypoxia and ischemia/reperfusion (I/R) injuries in neonatal rat cardiomyocytes and I/R rat hearts can induce apoptosis-related gene GADD153 overexpression, resulting in the down-regulation of CARP (Han *et al.*, 2005; Lee *et al.*, 2009). These studies demonstrated that hypoxia could down-regulate CARP expression in cardiomyocytes through GADD153. Conversely, CARP was found to be significantly increased in the swine model of transient ischemia (Depre *et al.*, 2001). These studies consistently suggested the protective role of CARP in cardiac ischemia injuries. However, a recent study revealed that overexpression of CARP enhanced cardiomyocyte apoptosis by promoting p53 activation and mitochondrial dysfunction in rodents (Shen *et al.*, 2015). The exact role of CARP on myocardial apoptosis needs further studies to clarify the initial results.

5.1.5 Heart failure

CARP mRNA and protein levels were markedly increased in the canine model of pacing-induced heart failure and human heart failure due to dilated or ischemic cardiomyopathy; however, it should be noted that this study only examined left ventricle-derived specimens (Zolk *et al.*, 2002). Subsequently, overcoming this limitation, it was shown that CARP down-regulated in atria and up-regulated in ventricles were evident in diastolic heart failure, while systolic heart failure results in up-regulation in both atria and ventricles occurring in the pig heart failure model. Interestingly, CARP presented a left-right asymmetric distribution with protein levels higher in the left as compared to the right ventricle (Torrado *et al.*, 2004; 2006). It still obscures the role and mechanisms of CARP asymmetric distribution in heart failure.

5.1.6 Atherosclerosis

It was recently reported that CARP was involved in inhibition of atherosclerotic lesion formation (de Waard *et al.*, 2003). CARP expression was observed in endothelial cells and quiescent intimal SMCs in human plaque. CARP-expressing SMCs are different from medical activated SMCs. However, CARP is not identified in quiescent SMCs in healthy vessels. Furthermore, TGF-β could activate CARP expression to inhibit the proliferation of vascular smooth muscle cells (VSMCs) (Kanai *et al.*, 2001). Collectively, these data suggest that CARP might be involved in the transition of activated SMCs into quiescent SMCs, thereby inhibiting the plaque progress.

5.2 Muscle disease

In the skeletal muscle, CARP expression is found to be low under basal conditions but could be induced in several circumstances such as exercise, muscular atrophy, amyotrophic lateral sclerosis (ALS), and other muscle pathologies (Carson *et al.*, 2002; Tsukamoto *et al.*, 2002; Nakada *et al.*, 2003a; 2003b; Barash *et al.*, 2004; Witt *et al.*, 2004; Hentzen *et al.*, 2006). In transient or definitive denervation-induced muscle atrophy and different muscular dystrophy models, the expression of CARP was persistently up-regulated, suggesting that it is a hub protein involved in the muscular pathological pathway. To further understand its contribution to muscle diseases, adenovirus fused with CARP coding sequence was injected into the tibial anterior muscle of normal mice. The results demonstrated no difference in muscle weight or histological appearance compared with the untreated mice, but slow-twitch fiber was reduced, suggesting that CARP overexpression in wild-type mice does not induce atrophy, but alters the fiber type composition (Laure *et al.*, 2009). In clinical studies, it has been reported that in congenital myopathies, CARP was expressed in severely damaged myofibers, but not detected in the central core disease (Nakada

et al., 2003a). These findings suggest that immunohistochemical evaluation of CARP may be helpful in the diagnosis of some congenital myopathies. Furthermore, CARP was found to be a sensitive and specific marker for rhabdomyosarcoma and it would be attributed in the diagnosis of rhabdomyosarcoma (Ishiguro *et al.*, 2008). However, the mechanism of CARP up-regulation and its exact role in muscular diseases remain obscure, possibly becoming a target for muscular disease therapy.

5.3 Other diseases

CARP mutation was identified in the total anomalous pulmonary venous return disease, a congenital heart defect in which pulmonary veins fail to enter the left atrium and drain instead into the right atrium or one of its venous tributaries. The mutation affected PEST-motif in CARP protein, thus enhancing the stability of the CARP protein (Cinquetti *et al.*, 2008). Increased CARP expression was also found in renal podocytes positively correlated with the severity of proteinuria in patients with lupus nephritis (Matsuura *et al.*, 2007) and cisplatin resistance in ovarian cancer chemotherapy (Scurr *et al.*, 2008), and CARP represents a novel target to sensitize tumors to platinum-based drugs (Lei *et al.*, 2015). Gene expression profiling following a crushing injury of the peripheral and central dorsal root ganglion neurons suggests that CARP expression is necessary for nerve regeneration (Stam *et al.*, 2007).

6 Conclusions

CARP is a multifunctional protein, which acts as a nucleus transcriptional co-factor negatively regulating cardiac genes and plays a significant role in different heart diseases, and is a component of sarcomere. Due to the important and multifunctional role of CARP, it could become a new diagnostic marker and therapeutic target of cardiovascular and muscle diseases after determining its role and understanding the mechanisms underlying the various roles it can assume.

Compliance with ethics guidelines

Na ZHANG, Xiao-jie XIE, and Jian-an WANG declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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中文概要

题　目：心锚重复蛋白的研究进展

概　要：心锚重复蛋白（CARP）是一个双重定位的蛋白，既可以在胞浆中作为肌节的结构组成蛋白，又定位于细胞核中作为转录共刺激因子调节其他基因的表达。研究发现CARP在多种心血管疾病及肌肉疾病中表达升高，但其在疾病中的作用尚存在争议。本文就CARP的研究进展进行综述，概述CARP的发现过程和结构，并对CARP在疾病中的作用的争议进行总结分析。

关键词：心锚重复蛋白；心血管疾病；心脏发育