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Role of Netrin-1 Beyond the Brain: From Biomarker of Tissue Injury to Therapy for Inflammatory Diseases

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Abstract

Acute kidney injury (AKI) is a common problem in the hospital setting and intensive care unit. Despite improved understanding, there are no effective therapies available to treat AKI. AKI is often diagnosed late and, therefore, identification of a biomarker that will facilitate early diagnosis of AKI and provide a prognosis is desirable. The recent identification of a novel biomarker, netrin-1, that is capable of diagnosing early during the course of kidney injury has created much interest in this molecule. In this paper, we review recent progress made in understanding netrin-1 as a biomarker and therapeutic molecule for acute and chronic kidney diseases. We also discuss both the usefulness of netrin-1 as a biomarker of other diseases and as a possible therapeutic agent and relevant patents for inflammatory diseases.

Keywords

Acute kidney injury; biomarker; cancer; chronic kidney disease; inflammation; netrin-1

INTRODUCTION

Organ damage due to ischemia is a major problem in the clinical setting. Currently, no effective treatment is available to prevent organ damage. Acute kidney injury (AKI) is a common and frequent complication and affects about 5% of all hospitalized patients with a mortality rate of greater than 50% in some populations. Despite advances in supportive care, this mortality rate has not decreased over the past 50 years. The high mortality of patients with AKI is not explained entirely by co-morbid conditions [1, 2]. Rather, AKI is an independent risk factor, increasing the risk of death by 10–15 fold compared to patients without AKI. For instance, in the United States, approximately 20.8 million people, or 7.0% of the population, are estimated to have diabetes and the incidence is growing. Diabetes has become the primary cause of end-stage renal disease (ESRD) in the US and the incidence of type 2 diabetes mellitus continues to grow in the US and worldwide. Approximately 44% of new patients entering dialysis in the US are diabetics [3, 4]. Early diagnosis of diabetes and intervention are critical in preventing the normal progression to renal failure seen in many type 1 and a significant percentage of type 2 diabetics. Currently there is no test available to diagnose early the development of diabetic nephropathy. Moreover, current therapeutics for diabetic nephropathy, mainly glycemic and blood pressure control, provide imperfect

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CONFLICT OF INTEREST

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protection against renal disease progression [5]. These points to the need for newer therapeutic agents that target primary mechanisms that contributes to the pathogenesis of diabetic nephropathy. The slow progress in the development of new diagnostic tests as well as treatment can be related to an incomplete understanding of the pathogenesis of diabetic nephropathy. Thus, new early diagnostic tests and therapeutic approaches are desperately needed for both acute and chronic kidney diseases.

Netrins and their Receptors

The netrins are a conserved family of laminin-related secreted proteins that were originally identified as axonal guidance cues [6]. In mammals there are three netrins (netrin-1, 3 and 4) and at least nine potential netrin receptors: Deleted in colorectal cancer (DCC) and neogenin (DCC family), Uncoordinated family member 5 (UNC5A-D or UNC5H1-4) (UNC5 families), Down syndrome cell adhesion molecule (DSCAM-1) [7] and $\alpha 6\beta 4$ and $\alpha 3\beta 4$ integrins [8, 9]. Additional netrin receptors have been postulated. Recent studies have shown netrin expression outside the nervous system including kidney [8, 10–12]. Using both *in vitro* and *in vivo* systems, netrin-1 was shown to play a role during angiogenesis, promoting developmental and therapeutic angiogenesis [13–15], cell migration [12], tissue morphogenesis [16,17], tumor growth [18, 19] and regulation of inflammation [10]. The netrin-1 receptor UNC5A-D has distinct domains, which include two immunoglobulin domains, two thrombospondin type I domains, one ZU-5 (domain found in Zona occludens) and one death domain. However, DCC has a different structure, containing 4 immunoglobulin domains, 6 fibronectin domains in the extracellular region and an ADD (addiction dependence domain) in the cytoplasm. In contrast to UNC5A-D, DCC does not have a classical death domain.

Netrin signaling is complex and is not always attractive or stimulatory. Binding of netrin-1 to DCC induces axonal attraction [20], whereas binding to the UNC5 receptor family causes repulsion [21, 22]. Although the importance of netrin and its receptors (DCC/UNC5B) in organ development, including neuronal and non-neuronal network systems, is well documented, the mechanisms by which signals are transduced downstream of the receptors are poorly understood. Netrin-1 binding to DCC on commissural axons causes recruitment of N-WASP (neuronal Wiskott-Aldrich syndrome protein), Pak1 (p21-activated kinase 1), Src kinases and FAK (focal adhesion kinase) to associate with the intracellular domain of DCC and induces activation of phospholipase C and the Rho GTPases Rac and Cdc42 [23]. These effectors collaborate to alter the axonal cytoskeleton to mediate turning in response to netrin-1.

Much less is known about the mechanism of UNC5 signaling. Tong *et al.* have reported that rostral cerebellar malformation (RCM), (now known as UNC5C), is tyrosine phosphorylated in transfected mammalian cells in the presence of DCC and netrin [24]. Co-expression of Src also induces RCM tyrosine phosphorylation. Recently, Li *et al.* showed that netrin induces UNC5C tyrosine phosphorylation and that DCC greatly enhances UNC5C tyrosine phosphorylation in response to netrin stimulation in transfected HEK-293 cells. In addition, FAK and Src family kinases were required for netrin-1 to stimulate this effect [25]. A more recent study has shown that netrin-1 induces interaction of UNC5B with the brain-specific GTPase PI 3-kinase enhancer long isoform (PIKE-L). This interaction triggers the activation of PtdIns-3-OH kinase (PI3K) signaling, prevents UNC5B's pro-apoptotic activity and enhances neuronal survival. Moreover, this process relies strongly on protein tyrosine kinase Fyn because PIKE-L is tyrosine phosphorylated in response to netrin-1, and the netrin-1-mediated interaction of UNC5B with PIKE-L is inhibited in Fyn-null mice [26]. Thus, PIKE-L acts as a downstream survival effector for netrin-1 through UNC5B in the nervous system. Recent studies also show that the caspase cleavage that occurs in the absence of ligand is critical for mediating apoptosis. Caspase cleavage releases a proapoptotic fragment

that basically encompasses the whole intracellular domain, which contains the ZU-5 and death domains. Indeed, it was shown that this fragment was sufficient to induce apoptosis when myristoylated and overexpressed in immortalized cells [27]. This suggests that a submembrane complex including UNC5H– addition/dependence domain (ADD) could be responsible for apoptosis induction. The ZU-5 domain and the death domain were shown to interact with proapoptotic proteins such as neurotrophin receptor p75 interaction MAGE homolog (NRAGE) and the serine-threonine death-associated protein kinase (DAPK), respectively [28]. The initial view of NRAGE implication in UNC5H1-induced cell death was that, in the absence of netrin-1, NRAGE is recruited by UNC5H1 through its ZU-5 domain. This interaction would block the inhibitor of apoptosis protein and, as a consequence, would activate caspases. Another possible role of NRAGE could be the activation of the c-Jun *N*-terminal kinase pathway, which also participates in apoptosis induction [29]. However, the implication of the ZU-5 domain in cell death was recently challenged with the determination of the three-dimensional structure of the intracellular domain of UNC5H2 [30].

It was shown that UNC5H intracellular domain displays two conformations: a closed conformation in which the ZU-5 and death domain interact together, the ZU-5 serving as a zip preventing the exposure of the proapoptotic death domain and an opened conformation in which ZU-5 does not interact with the death domain, allowing induction of apoptosis through this domain. It was proposed that in the absence of netrin-1, the death domain of UNC5H is released, interacts with DAPK, and allows its activation with DAPK, which occurs via a loss of its autophosphorylation [31]. One possible explanation for this loss of DAPK autophosphorylation could be the activation of a protein phosphatase and, along this line, it has recently been shown that DAPK is dephosphorylated by a protein phosphatase 2A (PP2A)-like phosphatase [32]. More recently, it was shown that UNC5H2/B recruits a protein complex that includes PR65 β and DAPK and retains PP2A activity. PP2A activity is required for UNC5H2/B-induced apoptosis, since it activates DAPK by triggering its dephosphorylation. Moreover, netrin-1 binding to UNC5H2/B prevents this effect through interaction of the PP2A inhibitor CIP2A to UNC5H2/B. Therefore, in the absence of netrin-1, recruitment of PP2A to UNC5H2/B allows the activation of DAPK via a PP2A-mediated dephosphorylation and that this mechanism is involved in the regulation of angiogenesis [33].

Discovery of Netrin-1 as a Biomarker of Kidney Injury

Netrin-1 was discovered as a kidney injury marker during spatial and temporal expression studies in the kidney after ischemia followed by reperfusion. Netrin-1 mRNA is expressed at the highest levels in kidney among the various organs studied so far [10, 34]. However, the protein level is not consistent with this highest mRNA expression level suggesting that there may be regulation at the level of protein production. Netrin-1 protein expression was localized in endothelial cells in normal kidney and very little or no protein expression was seen in tubular epithelial cells [34]. Within hours after reperfusion, netrin-1 protein expression appears in proximal tubular epithelial cells but, at the same time, the expression in vascular endothelial cells is down-regulated. Since netrin-1 is a secreted protein, it also appears in urine and can be quantified immediately after reperfusion. This led to the discovery of netrin-1 as an early diagnostic biomarker of kidney injury Table 1. Netrin-1 has a molecular mass of 72 KDa. Therefore, it is unlikely filtered by glomerulus under normal condition however netrin-1 may be filtered after injury as AKI is known cause changes in the filtration barrier. Therefore, part of urine netrin-1 might come from plasma as well. However, need further study to determine if plasma netrin-1 contributes to urinary netrin-1.

Initial studies suggested that netrin-1 was highly induced within a few hours after various injurious insults to the kidney, which include cisplatin, endotoxin, folate, and ischemia. Excretion of netrin-1 in urine peaks 6 hours after administration of these drugs or ischemia, whereas the currently used diagnostic test, serum creatinine, starts to rise 24–48 hr after injury [35]. These initial studies in animals demonstrated that indeed netrin-1 is induced from proximal tubular epithelial cells early in the course of kidney injury and can be used as a diagnostic biomarker of acute kidney injury.

Validation of netrin-1 as an early diagnostic biomarker of acute kidney injury:

Human studies—Preclinical studies demonstrated that netrin-1 protein from kidney tubular epithelial cells is up-regulated and appears in the urine early (within 1–3h) after murine renal ischemic injury as well as other forms of kidney injury [35]. Subsequently, a prospective study in human pediatric patients undergoing cardiopulmonary bypass (CPB) surgery was carried out to determine whether urinary netrin-1 levels could be used to predict the development of AKI early. The study included 26 patients who developed AKI (defined as a 50% or greater increase in serum creatinine after CPB) and 34 controls (age, race, and gender-matched patients who did not develop AKI after CPB). Patients with pre-existing renal insufficiency and nephrotoxin use were excluded. AKI was diagnosed only at 72hr after surgery using the traditional marker serum creatinine. In contrast, urine netrin-1 increased at 2h after CPB, peaked at 6h, and remained elevated up to 48h after CPB [36]. The predictive power of netrin-1 as demonstrated by area under the receiver operating characteristics curve for diagnosis of AKI at 2, 6, and 12 h after CPB was 74, 86 and 89% respectively. Also, by multivariate analysis, netrin-1 was independently associated with an increase in serum creatinine, cardiac pulmonary bypass time, length of hospital stay and number of AKI days. In addition, netrin-1 levels were significantly different between groups within AKI as well as between AKI and non-AKI groups even after stratification with pRIFLE criteria [36]. These results demonstrated that netrin-1 is an early, predictive biomarker of AKI after CPB.

Despite this promising and high predictive value of netrin-1 in pediatric patients, its usefulness as a biomarker in adult patients is not known. There are no prospective studies published with netrin-1 as an early biomarker of AKI in adults. However, random sampling from patients who had established renal failure due to sepsis, radiocontrast agent, drug-induced AKI and ischemic AKI showed much higher levels of netrin-1 as compared to healthy controls, suggesting that netrin-1 could be a universal tubular injury biomarker in adults as well [37]. The drawback of this study is that the urine samples were collected from patients with already established renal failure; therefore, it is not possible to determine whether the netrin-1 level increase is prior to the serum creatinine increase in the adult population.

Another area of kidney disease that requires biomarkers for early diagnosis is organ transplantation. Graft loss in kidney transplant patients is in part due in part to ischemia reperfusion injury. There are no validated biomarkers available for early diagnosis of future dialysis, graft dysfunction and eventual organ failure. Recent studies showed that netrin-1 is a good predictor for kidney transplant graft recovery and function. Netrin-1 levels in urine reflect functional recovery of the kidney in transplant patients. In that study, it was shown that all patients attained near normal functional recovery as reflected by a decrease in serum creatinine, which paralleled a decrease in urine netrin-1 levels, and by day 14, netrin-1 in urine was undetectable. One patient had sustained renal failure and showed high levels of netrin-1 in urine [37]. These results suggest that netrin-1 could be a useful biomarker for graft function and recovery. However, a multi-center prospective study has yet to be carried out to determine this possibility.

Netrin-1 and Chronic Kidney Disease

The diagnostic test currently used for chronic kidney disease (CKD) in clinical practice, i.e., increased albumin excretion rate, has significant limitations. Microalbuminuria is rarely found during the first five years following diagnosis of diabetes mellitus, suggesting that it is a sign of early glomerular damage rather than a marker for susceptibility to it. Furthermore, the presence of microalbuminuria by itself may not be an adequate indicator for disease progression in all diabetic subjects because of the observation that a number of type 1 diabetes patients revert to normoalbuminuria without treatment within a 5-year period [38]. Lastly, the occurrence of microalbuminuria in type 1 diabetes can already be associated with diabetic nephropathy lesions comparable to the ones found in overt diabetic nephropathy. Therefore, identification and validation of biomarkers for early diagnosis of diabetic kidney disease, i.e., before microalbuminuria develops, may help develop effective treatment for diabetic kidney disease. Although, diabetic nephropathy is classically considered as a glomerular disease, recent studies suggest that tubulointerstitial injury may precede apparent glomerulopathy [39]. This view was further supported by studies that show increased levels of tubular injury biomarkers such as kidney injury molecule-1 (KIM-1) and N-acetyl- β -D-glucosaminidase (NAG) in patients with type 1 diabetes [40, 41], and decreased levels of these biomarkers were associated with regression of microalbuminuria [41]. Similarly, increased levels of KIM-1 and neutrophil gelatinase associated lipocalin (NGAL) were seen in patients with established diabetic nephropathy [40].

Similar to KIM-1 and NGAL, the excretion of netrin-1 is increased in diabetes without microalbuminuria, which was further increased with microalbuminuria, and maximum concentration was found in diabetes with macroalbuminuria and renal dysfunction, Fig. (1). These results suggest that netrin-1 could be an early diagnostic biomarker of diabetic nephropathy. However, a longitudinal long-term study needs to be carried out to determine whether netrin-1 can predict accurately the development of nephropathy in diabetic patients.

Looking beyond the biomarker of kidney diseases—Recent studies suggest that netrin-1 is up-regulated in other disease models where hypoxia and tissue injury are prevalent, which includes cancers of various organs [19, 42, 43], and either inhibition of netrin-1 activity or down-regulation of netrin-1 expression induced apoptosis in tumor cells, leading to tumor regression. More recently, using enzyme linked immunosorbent assay (ELISA), netrin-1 levels in plasma were quantified in many cancer patients and their controls. Plasma levels of netrin-1 were significantly elevated in patients with adenocarcinoma of breast, prostate, and liver, renal cell carcinoma, and brain tumors (pituitary adenoma, meningioma, glioblastoma) as compared to their corresponding control patients. Elevated levels of netrin-1 were not universal in all cancers. Although tissue expression was shown to be increased in adenocarcinoma of lung, pancreas and colon cancers, plasma level of netrin-1 was not increased [44–46].

Conventional receiver operating characteristic (ROC) curves analysis for cancer versus no cancer shows a high level of sensitivity and specificity for cancer diagnosis. However, netrin-1 is not specific to a particular type of cancer. The lowest sensitivity was seen for lung and highest sensitivity was seen for prostate adenocarcinoma. It is interesting to note that plasma levels of netrin-1 are not increased in AKI but rather are down-regulated ([47] and unpublished observation). Inflammatory cytokines are known to suppress netrin-1 expression in vascular endothelial cells [10]. Inflammation is a hallmark of acute kidney injury. Therefore, inflammatory cytokines may also play a role in the down-regulation of netrin-1 in the circulation immediately after reperfusion of the kidney. In contrast to netrin-1 plasma levels in cancer, it is interesting to note that plasma levels of netrin-1 are not increased in AKI. Since netrin-1 level in the circulation is significantly increased in many

cancers, plasma netrin-1 can be used as a biomarker of many human cancers, and may be useful as a prognostic tool as well.

Netrin-1 as a Regulator of Inflammatory Kidney Diseases and Beyond: Will it be Useful as an Effective Therapeutic Agent for Kidney Injury?

Although netrin-1 was identified as a neuronal guidance cue, directing neurons and their axons to targets during development of the nervous system [48], this is unlikely to be its only function, since expression studies have shown that netrins are widely expressed outside the nervous system, including in vascular endothelial cells [10, 34]. Vascular endothelial cells represent a critical barrier for leukocyte activation and migration into organs by producing repellent factors to leukocytes such as netrin-1. Down-regulation of netrin-1 during organ injury may exacerbate inflammation [10, 34,49]. In fact, administration of recombinant netrin-1 or overexpression of netrin-1 in kidney suppressed inflammation and apoptosis *in vivo* [10, 34,50]. In addition, kidney-specific overexpression also protected against tissue injury due to ischemia reperfusion and cisplatin [50, 51]. The netrin-1 receptor UNC5B is expressed on leukocytes and mediates netrin-1 inhibitory effects on leukocyte migration in a peritonitis model [10] and ischemia reperfusion injury of the kidney [34, 47] and other tissues [49, 52]. *In vitro* studies have demonstrated that netrin-1 potentially inhibits monocyte, lymphocyte, and granulocyte migration [10]. The inhibition of leukocyte migration was dose-dependent and mediated through the UNC5B receptor both *in vitro* [10] and *in vivo* [47]. Adenosine receptor 2B (A2BAR) was proposed as a receptor for netrin-1 and shown to mediate netrin-1 protective effects in acute lung injury and peritoneal inflammation [53, 54]. However, the direct link between A2BAR and netrin-1 has never been demonstrated. Moreover, the phenotypes of netrin-1 and A2BAR knockouts do not match [55, 56]. Whether netrin-1 mediates part of its protective effect through A2BAR through an increase in adenosine production has never been examined. The role of other netrin-1 receptors in mediating the suppressive effects on the inflammatory cells is not clear.

Inflammation is a major contributing factor in many ischemic and toxic organ injuries as well as in autoimmune diseases [49, 57–60]. Molecules that suppress activity of inflammatory cells without killing them would be desirable for treatment purposes. This approach will not have problems with associated chronic immune deficiency, which dispose the individual to infection. Therefore, netrin-1 appears as an attractive anti-inflammatory molecule that also supports endothelial cell function. However, rigorous testing and clinical trials in large animals need to be carried out before translating it as an effective therapeutic molecule of inflammatory diseases such as ischemic AKI.

Regulation of Netrin-1 Expression

The regulation of netrin-1 in different tissues and disease states is not well understood. It was shown that a NFkB-mediated transcriptional mechanism is responsible for increased netrin-1 expression in inflammatory bowel disease [61] of the gut. However, another study showed that inflammatory cytokines actually down-regulated netrin-1 expression [10, 53] but the mechanism was not determined. It was also demonstrated that hypoxia inducible factor-1 (HIF-1) increases netrin-1 transcription in intestinal epithelial cells in response to hypoxia [49], although the increase is modest at most. Moreover, netrin-1 transcript levels are down-regulated in response to ischemia in kidney and kidney epithelial cells [34, 62]. At same time, however the expression of netrin-1 protein is induced several fold, suggesting a post-transcriptional mechanism may exist. This view was supported by a recent study in kidney epithelial cells treated with various injurious agents Fig. (2). Pervanadate treatment highly induced netrin-1 production independent of transcription and mRNA stability in the kidney tubular epithelial cells [62]. Increased production of netrin-1 in tubular epithelial cells was mediated through the ERK and Akt pathways, which increased the translation of

netrin-1 mRNA. It was also demonstrated that cisplatin, a high level of protein in the medium, endotoxin, and hypoxia induced netrin-1 protein production by a post-transcriptional mechanism and could be inhibited in the presence of the MEK2 inhibitor U0126 [62]. Scanning of the 5'-UTR with the UTRdb database identified an internal ribosome entry site (IRES) (sequence 133–220 of 5'-UTR) and a K-box sequence at the 3'-UTR (sequence 2650–2657); these two elements are known to increase translation and decrease stability of mRNA, respectively. Both increased mRNA translation and increased degradation (decreased stability) was noted in recent studies, suggesting that both elements might play an important role in netrin-1 mRNA translation. Further genetic studies using the 5'-UTR and 3'-UTR cloned upstream of luciferase or other genes will enable us to determine the influence of these elements.

CURRENT & FUTURE DEVELOPMENTS

Biomarker discovery and validation for AKI has accelerated significantly in the past decade. However, standardization of biomarker measurements and determining which biomarker or combination of biomarkers will be useful for accurate diagnosis of AKI at the earliest time is not clear. Moreover, almost all biomarker studies and validation have been based on serum creatinine levels rather than a biomarker itself as a reflection of disease process. Therefore, more studies are required before drawing conclusions on the direct relation of a biomarker with histologic changes or disease process. This is important since a biomarker that is more sensitive than serum creatinine may lead to over-diagnosis or diagnosis of sublethal tissue injury. It is also possible that those patients who show increased levels of a biomarker in urine without any obvious alteration in serum creatinine may have underlying kidney injury, and several such episodes may lead to the development of chronic kidney disease. It is very difficult to examine this possibility in the clinic. Measuring a biomarker for specific tubular segments also has been discussed. Although this may lead to a better understanding of the injury process, whether it will be useful in treating overall disease is not clear.

Recently, several patents are awarded on netrin-1 use as diagnostic marker various disease and use of netrin-1 for treating diseases which suggest the bright future for the development of diagnostic test and/or treatment option based on netrin-1. Few of these patents on netrin-1 are listed in Table 1 [63–65]. Netrin-1 is a multifunctional, laminin-related, secreted protein highly induced in response to kidney injury and is excreted in urine. *In vitro* and *in vivo* studies have shown that netrin-1 plays an important role in the regulation of inflammation, apoptosis and cell migration. However, the mechanism as to how netrin-1 protects the kidney from various injurious insults is not completely understood. Identification of netrin-1 as a biomarker of both acute and chronic kidney disease suggests that a common mechanism may exist. In contrast to an earlier report, netrin-1 is regulated at the translational level in the kidney in response to injury Fig. (2). Netrin-1 is a promising early biomarker of acute and chronic kidney injury, and further prospective studies will enable us to advance our understanding of the kinetics of netrin-1 induction in human diseases. It will also be interesting to see whether netrin-1 induction is dependent on age and regeneration capacity of the kidney in the future. This might unravel the possibility that netrin-1 may initiate a regeneration process immediately after injury. There are several limitations of currently available studies on netrin-1. First, it is a single-center pilot study in a small population of pediatric subjects with congenital heart defects undergoing elective cardiac surgery. Thus, these results will need to be validated in a larger multicenter studies in diverse population. Second, netrin-1 assay used has limited external validity, limited analytical and assay standardization. Therefore, developing a robust uniform standardized assay for clinical platform is required.

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Fig. 1.

Netrin-1 in diabetic human urine is increased with renal injury. Urine samples from non-diabetic controls, diabetic patients (DM) with or without microalbuminuria (MAU), and with or without abnormal renal function (RF), as indicated (pooled urine from 10 patients in each group) and 2 μ g creatinine equivalent urine was loaded on to 10% polyacrylamide gel, and Western blot analysis was carried out for netrin-1. Netrin-1 level is increased in a stage-dependent manner and was highest in patients with microalbuminuria and renal dysfunction.

Fig. 2. Netrin-1 synthesis is increased in response to tubular epithelial cell injury through an ERK and PI3K-mediated translational increase of its mRNA. Netrin-1 is secreted into the tubular lumen and excreted in urine and thus used as biomarker of kidney injury.

Table 1

Examples of Recent Patents on the Use of Netrin-1 as Biomarker of Diseases.

Patents on netrin-1 Related to Biomarker and Inflammation	
1	Ramesh G: Netrin-1 as a biomarker of injury and disease. US8052960 (2011)
2	Kinane TB, Komatsuzaki K and Ly N: Netrin compositions and methods of using the same. US0025335 (2006)
3	Mehlen P, Bernet A and Fitamant J: Screening For Anti-Cancer Compounds Using Netrin-1 Activity. US00226458 (2009)