INTRODUCTION

Neurodegenerative disease (NDD) is a general term that encompasses several brain disorders categorized by the gradual deterioration of various neuronal systems and includes Alzheimer’s disease (AD), Parkinson’s disease (PD), Huntington’s disease (HD), amyotrophic lateral sclerosis (ALS) among others. Since the most pathologic sites in NDD are in the brain, where accessibility to obtain a biopsy sample is difficult, accurate diagnosis of NDD is challenging. Therefore, the availability of reproducible and reliable biomarkers to diagnose such diseases is more critical than ever. In addition, biomarkers could be used not only to diagnose diseases but also to monitor the development of disease therapeutics. Urine is an excellent biofluid that can be utilized as a source of biomarker to diagnose not only several renal diseases but also other diseases because of its abundance in invasive sampling. However, urine was conventionally regarded as inappropriate as a source of biomarker for neurodegenerative diseases because it is anatomically distant from the central nervous system (CNS), a major pathologic site of NDD, in comparison to other biofluids such as cerebrospinal fluid (CSF) and plasma. However, recent studies have suggested that urine could be utilized as a source of NDD biomarker if an appropriate marker is predetermined by metabolomic and proteomic approaches in urine and other samples. In this review, we summarize such studies related to NDD.

Key words: Urine, Biomarkers, Parkinson’s disease, Alzheimer’s disease

DETECTION METHODS

A good biomarker and its assay for diagnostic purposes should be highly sensitive and selective as well as simple, reproducible and inexpensive. However, it is practically almost impossible to satisfy

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Urinary Biomarkers for Neurodegenerative Diseases

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Global incidence of neurodegenerative diseases (NDDs) such as Alzheimer’s disease (AD) and Parkinson’s disease (PD) is rapidly increasing, but the diagnosis of these diseases at their early stage is challenging. Therefore, the availability of reproducible and reliable biomarkers to diagnose such diseases is more critical than ever. In addition, biomarkers could be used not only to diagnose diseases but also to monitor the development of disease therapeutics. Urine is an excellent biofluid that can be utilized as a source of biomarker to diagnose not only several renal diseases but also other diseases because of its abundance in invasive sampling. However, urine was conventionally regarded as inappropriate as a source of biomarker for neurodegenerative diseases because it is anatomically distant from the central nervous system (CNS), a major pathologic site of NDD, in comparison to other biofluids such as cerebrospinal fluid (CSF) and plasma. However, recent studies have suggested that urine could be utilized as a source of NDD biomarker if an appropriate marker is predetermined by metabolomic and proteomic approaches in urine and other samples. In this review, we summarize such studies related to NDD.

Key words: Urine, Biomarkers, Parkinson’s disease, Alzheimer’s disease

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all these criteria. Therefore, the identification of better biomarkers is essential and related studies are being conducted extensively. Currently, proteomic and metabolomic studies to identify whole target material in the samples are possible because of the advancement in liquid chromatography (LC) and mass spectrometry (MS), enabling the rapid and unbiased identification of biomarker candidates.

There are several tools to analyze bioactive substances in urine depending on the chemical identity of the target material. MS provides the best sensitivity, selectivity and quantitative identification capabilities to analyze the most bioactive compounds from proteins to simple metabolic chemicals. Sometimes, MS can be used with LC or gas chromatography (GC). However, these apparatuses are very expensive and require skilled manpower to run them. Enzyme-linked immunosorbent assay (ELISA) is also a sensitive method to quantitatively detect various metabolic substances. Although a specific antibody against the target substance is a prerequisite for ELISA, if the target is decided and the antibody is available, ELISA can be easily done even in a small laboratory. However, to designate a specific substance as a biomarker, a high-throughput method or an educated guess is necessary. Therefore, despite its cost, MS-based proteomic analysis and metabolic profiling of urine from patients with a specific disease is gaining prominence to identify a novel disease-specific biomarker [14-16]. If the target material is nucleic acid, next generation sequencing or small RNA sequencing can be a tool for a high-throughput screening method [17]. However, there is no such study with urine yet, although miRNA studies using CSF have been reported [18, 19]. When the target material is a specific protein, western blot analysis can be a simple and cost-efficient method although accurate quantification of the result could be difficult. This can be applied to specific Aβ peptides or phospho-Tau for AD and monomeric of oligomeric forms of α-synuclein for PD because of the well-known causal relationship between these proteins and the corresponding disease [20, 21]. In addition, a recent study reported the use of an antibody labelled with fluorescent dye to detect Aβ1-42 in ELISA kits [22].

Low levels of urinary bioactive material make biomarker detection more challenging. However, it can be partially solved by increasing the concentration of urine by proper methods such as microfiltration or fractionation of active material [13, 23]. Another method to solve this problem is to utilize urinary exosomes [8, 11, 24-26] which contain various cellular metabolites such as proteins, nucleic acids and lipids whose compositions reflect the physiology of cells from which they were originated. Such metabolites in urine exosomes are generally higher than their urinary concentrations [27]. Therefore, urinary exosomes may be critical sources to develop a specific, non-invasive and cost-effective disease diagnostic and monitoring tools [28]. Accordingly, urinary exosomes were investigated for their diagnostic and therapeutic potentials in NDDs [1]. In fact, increased levels of DJ-1 and phosphorylation of S1292 (p-S1292) LRRK2 were observed in the urinary exosomes of male PD patients [8, 12]. In addition, systemic approaches with proteomic analysis of urine exosomes from patients have identified SNAP23 and calbindin as novel PD biomarkers for PD [6] and higher levels of Aβ1-42 and p-S396-Tau for AD [29].

In the following sections, we briefly introduce recent efforts to identify urine biomarker candidates to diagnose NDD that are present in specific bioactive substances (e.g., metabolites, proteins/peptides, miRNAs and DNAs) and in exosomes (Table 1). The purpose of this review is brief, but extensive introduction on this subject, and therefore, more detailed information is available in the references listed here. In addition, although NDD includes AD, PD, ALS and HD and others, we limit our discussion to PD and AD as these are the two most common NDDs and their major pathogenic mechanisms, accumulation of misfolded/unfolded proteins and increase of oxidative stress, are common to most of the NDDs [30].

**Metabolites**

Metabolomics is one of the emerging -omics that aims to identify most metabolites within a target sample and their change. Luan et al. [14] analyzed 157 urine samples, including 92 samples from PD patients and 65 samples from normal control subjects with GC-MS and LC-MS. The resulting metabolic profiles identified 18 metabolites which are differentially expressed between PD and controls [14].

Dopamine and its metabolites were detected in urine as well as in CSF and plasma [31]. Urinary dopamine was higher in PD patients under L-DOPA treatment as expected [32]. Besides, the concentrations of 5- and 6-hydroxydopamine in the urine from L-DOPA treated PD patients were also higher than those from non-treated patients and from normal controls [33], discouraging the use of dopamine as a PD biomarker. A recent report identified kynurenine as a urinary PD biomarker [34]. The kynurenine pathway had been suggested to correlate with PD mechanism [35] and the alterations of kynurenine levels in CSF and blood from PD patients have been reported [36, 37].

APP transgenic mouse, a well-established AD animal model, was widely used to study the metabolomic changes related to AD [38]. A significant increase of 5-hydroxyindoleacetic acid (5-HIAA), the major metabolite of serotonin, has been reported in the urine of AD model mice [39]. Another promising candidate is urine formaldehyde which shows inverse correlation with Mini Mental State Examination (MMSE) scores [40-42]. The human urine study [43]
Urine as a Source of Neurodegenerative Diseases Biomarker

suggested several metabolites in amino acid metabolism such as L-glutamine, 5-L-glutamylglycine and phospholipid metabolites as novel AD biomarkers. Oxidative compounds are also good candidates for AD biomarkers because, like PD, oxidative stress is a major causative factor for AD. One recent study reported significantly higher urinary levels of lipid peroxidation compounds in AD compared to control cases [44].

However, several metabolomic studies have suggested that some

| Target substance | Disease | Detection method | Target | Number of case | Conclusion* | Reference |
|------------------|---------|------------------|--------|----------------|-------------|-----------|
| Metabolites      | AD      | UPLC coupled with quadruple time-of-flight MS | Mouse | 30 | 30 5-hydroxyindoleacetic acid† and change of other 25 metabolites | [39] |
|                  |         | LC–MS/MS targeting 3-HPMA, ELISA for AC-Acro | Human | 32 | 74 3-HPMA/Creatinine† | [85] |
|                  | PD      | Head-space GC/MS Non-targeted metabolic profiling and ELISA for kynurenine | Mouse | 38 | 34 1-octen-3-ol↓ | [86] |
|                  |         | LC–MS base | Human | 106 | 104 Cortisol↑, 11-deoxycortisol↑, 21-deoxycortisol↑, histidine↑, urocortin acid↑, imidazoleacetic acid↑, hydroxy-phenylacetic acid↑ | [15] |
|                  |         | LC–MS & GC–MS base | Human | 92 | 65 Change of 18 metabolites (alanine, leucine, isoleucine, aminobutyric acid, hydroxytryptophan, creatinol, etc.) | [14] |
| Nucleic acids    | AD      | Ultra-performance LC–tandem MS | Human | 53 | 27 8-OHdG↑ and 8-OHdG/2dG↑ | [44] |
|                  | PD      | ELISA for 8-OHdG | Human | 72 | 48 (8-OHdG)/creatinine↑ | [76] |
| Protein          | AD      | LC–MS/MS | Mouse | NS | NS Kallikrein-1 (↑ or ↓ depending on mouse age) | [7] |
|                  |         | ELISA for AD7c-NTP | Human | 66 | 134 AD7c-NTP↑ | [52, 54, 56, 57, 88] |
|                  | PD      | LC–MS/MS | Human | 18 | 18 Change of 73 proteins (≥2-fold↑) and 36 proteins (<-0.5-fold↓) | [16] |
|                  |         | 2 gene expression data of brain samples → iTRAQ | Human | 20 (urine) | 20 (urine) SPP1↓, GSN↑, and IGFBP7↑ | [51] |
|                  |         | Scalable and sensitive MS | Human | Discovery:28 replication:51 Cohort 1: 35 HC, 16 NMC, 40 iPD, 28 LRRK2 PD; Cohort 2: 26 HC, 37 NMC, 29 iPD, 23 LRRK2 PD | Discovery:22 replication:57 SNAP23↑, and calbindin↑ | [6] |
|                  |         | ELISA for oligomer, filament, and total α-syn Western blot | Human | 21 | 11 Specific filament form of α-synuclein↑ | [13] |
|                  |         | Western blot | Human | 26 | 21 DJ-1↑ (in Male only) | [8] |

*Some targets are omitted because of space limit.
NS, not specified; 2dG, 2’-deoxyguanosine; 3-HPMA, 3-HPMA-3-Hydroxypropyl mercapturic acid; Cre, creatinine; AC-Acro, amino acid-conjugated acrolein; 8-OHdG, 8-hydroxy-2’-deoxyguanosine; AD7c-NTP, AD-associated Neuronal Thread Protein; EV, Extracellular Vesicle; ELISA, Enzyme-Linked ImmunoSorbent Assay; HC, Healthy Controls; NMC, Non-Manifesting LRRK2 G2019S Carriers; iPD, idiopathic PD patients; LRRK2 PD, manifesting PD patients with LRRK2 G2019S.
biomarker candidates are not disease-specific, indicating that they were also found with other neurodegenerative diseases in addition to the originally targeted disease [45]. This is predictable when one considers that the molecular mechanisms associated with pathogenesis of NDDs are frequently overlapped.

**Proteins**

Approximately 20% of urinary proteins were derived from blood after renal filtration and proteins less than 20 KDa can cross the kidney filtration barrier although most of them are reabsorbed. However, proteins bigger than 100 KDa had been also detected in urine [46]. This implied the potential of urinary proteins to diagnose diseases in addition to renal diseases [47].

Efforts to identify biomarkers for AD or PD are conducted in two directions. One is to investigate whether well-known disease-specific proteins (e.g., APP/Aβ or Tau for AD, and α-synuclein, LRRK2 or DJ-1 for PD) are present in different levels and another is a systemic approach such as proteomics to identify proteins showing different levels between patient and control samples. Specific forms of Aβ peptides are neurotoxic and their presence in the brain is regarded as an early indicator of AD [48]. Oligomeric or high concentration of α-synuclein has been well-known to contribute to PD progression [49]. Monomeric or oligomeric forms of α-synuclein were extensively investigated for their potentials as biomarkers in CSF and plasma [50]. However, the results were inconsistent. In contrast, there are few reports on urinary α-synuclein, which might be due to low concentration of α-synuclein. Very recently, we observed the presence of α-synuclein in the urine after 10-fold concentration of urine through microfiltration and reported the significant increase of filamentous α-synuclein in PD urine samples [13]. In addition, we have reported the increase of DJ-1 in urine from male PD patients [8].

Several MS proteomics approaches could identify gene ontology terms showing significant differences between patient and control samples via gene ontology enrichment analyses in addition to identification of an individual biomarker candidate(s). For example, matrix metalloproteinase signaling, lipoprotein metabolism and heat shock protein 90 signaling pathways were related to AD [16]. An interesting study identified differentially expressed gene in AD brain through computational analysis and then verified the differences in the levels of urinary proteins encoded by these genes [51]. They found that osteopontin, gelsolin and insulin-like growth factor-binding protein 7 were differentially present in the urine of AD patients [51]. This type of approach which identifies target substances in the brain or CSF and then confirms their differences in urine between patients and healthy controls may provide a new direction to identify novel urine biomarkers. Increased levels of Alzheimer-associated neuronal thread protein (AD7c-NTP) in urine are detected in the AD samples in several independent studies [52-54]. A company called Nymox had obtained European approval to sell an Enzyme Immunoassay (ELA) kit to detect urinary NTP levels to diagnose AD, but there was a controversy [55] as an independent study found that the test tended to show low specificity and sensitivity. However, the potential of AD7c-NTP as a urine biomarker for AD as well as mild cognitive impairment (MCI) is still being actively pursued [56, 57].

One of the most recent and extensive PD proteomic profiling has been reported by Winter et al. [58]. They analyzed more than 200 urine samples from two independent cohorts consisting of LRRK2 WT healthy controls, non-manifesting carriers of LRRK2 G2019S, a major genetic factor for PD pathogenesis, idiopathic PD patients and PD patients with the LRRK2 G2019S mutation through scalable and sensitive MS-based proteomics. Their results indicated that urinary proteomics was sufficient to distinguish the presence of either LRRK2 mutation or the disease, confirming that urinary proteomics has the potential to identify a novel PD biomarker [58].

The possibility of different levels of modified proteins such as phosphorylation or oxidation was also investigated. Hyperphosphorylation of Tau is critical for AD pathogenesis and a higher level of phosphorylation of S396 (p-S396) in Tau in the urinary exosomes of AD patients was reported [29], p-S1292 LRRK2 is critical for its PD pathogenesis and higher levels of p-S1292 LRRK2 in the urinary exosomes of PD patients were reported [12]. Similarly, a higher level of oxidized DJ-1 in the urine of PD patients was also reported [59].

**RNAs and DNAs**

miRNAs are small non-coding RNAs that regulate the expression of their target genes [60]. Various miRNAs have been reported to alter the expression of genes related to PD such as mitochondria dysfunction, α-synuclein regulation, oxidative stress and neuroinflammation [61]. Their presence in CSF, saliva, plasma and urine promoted the investigation of miRNAs for their biomarker potential for NDDs [3]. One systemic study searched all miRNAs reported to be significantly deregulated in the blood and compared them to the miRNAs deregulated in the brain at Braak Stage III of AD. Using this approach, 10 miRNAs were identified to be dysregulated in early AD stage [62]. Other studies also proved the potential of circulating miRNAs as NDD biomarkers [17, 28]. It is an additional advantage that urinary miRNAs are relatively stable even after 10 cycles of freezing and thawing [63]. Although their low concentration in the urine can be a disadvantage [64], it can be overcome by utilizing miRNAs in the urinary exosome where bio-
active substances are relatively concentrated. However, there are few reports to investigate urinary miRNAs as NDD biomarkers. Urinary mRNAs are considered as inappropriate biomarkers because of their instabilities. However, the instability issue can be circumvented by the pretreatment of urine by the addition of guanidinium thiocyanate [65], or by their isolation from urinary exosomes or cells present in the urine. Accordingly, specific mRNAs as well as their splice variants were reported as specific biomarkers of muscular dystrophies [66] and renal fibrosis [67]. Urinary genomic DNAs (gDNAs) mostly were derived from cells secreted to the urines and mutations in such gDNAs were used to detect bladder cancer [68]. A recent approach showed that purification and high-throughput sequencing of cell free urinary DNAs are possible [69]. Detection of differences of gDNAs from the urine samples of the diseases and the healthy controls could be possible, if any.

The mitochondria have their own DNA to encode several critical enzymes for ATP synthesis and change of mitochondrial (mt) DNA has been suggested as AD and PD biomarkers. Leakage of mtDNA is associated with mitochondrial impairment. Cell-free mtDNA can be secreted in the biofluid and detected with specific primer sets through PCR. Various mtDNA mutations in the brain were reported as risk factors of AD [70]. Decrease of cell-free mtDNA copies has been also observed in PD CSF samples [71]. This was confirmed by Podlesniy et al. and they further showed that the copy number of mtDNA in CSF was significantly higher in PD patients with G2019S mutation group than the idiopathic PD or healthy G2019S carrier group [72]. It is yet to be investigated whether these changes are maintained in urine cell-free mtDNAs.

Oxidative stress is a major cause of both PD and AD and oxidized DNA can be generated during disease progression. 8-hydroxy-2’-deoxyguanosine (8-OHdG) is the most studied form of oxidized DNA and its urinary level has been used to measure oxidative stress in human [73]. Consequently, significantly higher levels of 8-OHdG in the urine of PD [74] and MCI-AD [75] cases were observed. In addition, the level of urinary 8-OHdG progressively increased as PD advanced [76]. Interestingly, the concentrations of urinary 8-oxo-7,8-dihydroguanine (8-oxoGua) and miR_6778_5p in samples of workers exposed to organic solvents showed a significant negative association [77], suggesting a possibility of similar approach to combine two different factors in NDD urines.

### CONSIDERATIONS

There are several points to consider before the practical application to screen urinary NDD biomarkers. First, quality control of the urine sample is critical because urinary proteins are easily affected by complicated factors such as sampling time, medicine and diet, especially in human samples. Before analysis, urine samples such as proteinuria or lipiduria should be excluded because such conditions may suggest a kidney problem [47]. For urinary analysis, the first urine in the morning is preferred. Several studies tested for consistency of urine proteins and metabolites, after repeated cycles of freezing and thawing or sampling at different times [8, 78-80]. Their results implied that most, if not all, bioactive materials are relatively stable after several cycles of freezing and thawing, but the sampling time mostly matters [63, 81]. However, if there is no absolute reason to obtain the first urine in the morning, sampling the urines at convenient times may be more practical because collection of the first urine in the morning needs additional efforts and limits cases of patients who can contribute the urine. In addition, a standard protocol for the processes to collect, handle and store specimens should be established before analysis to reduce the inconsistency of results between studies. Secondly, some candidates were identified in other diseases as well as the target disease, suggesting that they might be involved in the NDD progression process itself rather than the disease-specific [45, 75, 76]. Therefore, when a biomarker candidate is identified in the urine, it is important to further confirm it in other samples or different diseases. Thirdly, a combination of multimodal biomarkers from urine as well as other biofluids might provide a better selective window. For example, addition of certain miRNA levels to Aβ42:T-tau measures in CSF improved classification of AD [18]. Similarly, a combination of levels of serum BDNF and urine AD7c-NTP in mild cognitive impairment (MCI) cases increases its prediction to their AD evolution [9]. For this purpose, before IRB application, planning analysis with both urine and other biosamples such as plasma or CSF might be desirable. Finally, the selection of proper normalized controls is critical. Although house-keeping genes such as GAPDH or β-actin are generally used as normalized genes for most western blot or PCR analysis, there are few reports on this subject, and moreover, the use of GAPDH as a urinary normalizer was contradictory [65, 81]. If the intact urine is used for analysis without any further process, the used volume itself can function as a normalizer [71, 72]. Based on this, it is desirable that processing of urine samples before analysis is minimal.

We recommend other references for more detail reviews [6, 44, 82-84].

### CONCLUSION

Novel biomarkers with higher specificity and sensitivity that can be easily tested at a reasonable cost are urgently needed for NDDs. Since urine can be invasively sampled in abundance, it can
be a good biofluid to identify biomarkers. Recent technological advances allow for the systematic, holistic, and unbiased characterization of alterations in RNAs as well as genes, proteins and metabolites in the urine associated with the identification of novel biomarkers and disease conditions, making these approaches more promising. There are even a few biomarkers such as urinary formaldehyde and 8-OHdG showing correlation with severity of the diseases [42, 76], suggesting their potential as markers to predict the therapeutic effect. However, it is required to confirm a candidate through independent studies.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Urine as a Source of Neurodegenerative Diseases Biomarker

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