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MOLECULAR CHARACTERIZATION OF MICRORNA INTERFERENCE AND ARISTOLOCHIC ACID INTOXICATION FOUND IN UPPER TRACT UROTHELIAL CARCINOMA IN PATIENTS WITH BALKAN ENDEMIC NEPHROPATHY: A SYSTEMATIC REVIEW OF THE CURRENT LITERATURE

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ABSTRACT

The term "aristolochic acid nephropathy" (AAN) is used to include any form of toxic interstitial nephropathy that is caused either by ingestion of plants containing aristolochic acids (AA) or by the environmental contaminants in food such as in Balkan endemic nephropathy (BEN). Aristolochic acid (AA) intoxication is strongly associated with the development of upper tract urothelial carcinoma (UTUC); however, the underlying molecular mechanism remains to be defined. MicroRNAs (miRNA) regulate several biological processes, including cell proliferation, differentiation, and metabolism, acting as oncogenes or tumor suppressors. A unique miRNA expression profile suggested that miRNAs could function as regulators in UTUC developmental processes.

This review aimed to summarize data available in the literature about underlying molecular mechanisms leading to the expression of miRNAs in AA-UTUC patients with BEN. Strong correlation in AA-UTUC has a distinctive gene alteration pattern, AL-DNA adducts, and a unique tumor protein (TP53) mutational spectrum AAG to TAG (A: T→T: A) transversion in codon 139 (Lys → Stop) of exon 5 activates the p53 tumor suppressor protein. Further, p53 protein is responsible not only for the expression of miRNAs but also acts as a target molecule for miRNAs and plays a crucial function in the AA-UTUC pathogenicity through activation of cyclin-dependent kinase (CyclinD1) and cyclin protein kinase 6(CDK6) to support cell cycle arrest. This study, proposed a molecular mechanism that represented a possible unique relationship between AA intoxication, miRNAs expression, and the progression of UTUC in patients with BEN.

Key words: aristolochic acids; Balkan endemic nephropathy; microRNA, upper tract urothelial carcinoma

INTRODUCTION

Balkan endemic nephropathy (BEN), initially described in the 1950s, is a chronic renal nephropathy disease that affects people that live in the alluvial plains along the tributaries of the Danube River [1]. Notable, the highest prevalence of BEN is observed in Serbia, Bulgaria, Romania, Bosnia and Herzegovina, and Croatia [2-6]. This devastating slowly progressive disease, which starts in the fifth decade and develops into terminal renal failure, urothelial carcinomas, or both, in the sixth or seventh decade of life, still represents an important medical, social, and economic burden for all countries harboring [7,8]. The number of patients undergoing dialysis remains unchanged; however, newly diagnosed cases seem to be shifting to older ages, pointing to lower exposure [9, 10]. On the other hand, despite several similar risk associations between all urothelial carcinomas (UC) such as tobacco smoking, genetic predisposition, chemical exposure, and increasing age, a strong association exist only between BEN and upper tract urothelial carcinoma (UTUC) [11-14]. The high prevalence of UTUC is an indispensable characteristic of BEN and the incidence of UTUC is significantly higher, even 100 times in areas where BEN is endemic than in no endemic regions [15, 16]. About 30–40% of the affected individuals develop UTUC, which are mostly papillary carcinomas

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and are the most common causes of death in BEN patients [17]. This retrospective study aimed to offer a narrative literature review using a different type of published omics data to understand the molecular mechanism underlying the nephropathy effect of aristolochic acid, (AAN) as the most important environmental factor for BEN/UTUC. This study pinpoints the interaction between epigenetic and environmental factors to understand the etiology, pathogenesis, and treatment of UTUC in patients who originated from areas where BEN is endemic.

Aristolochic acid (AA) intoxication and UTUC

Aristolochic acid (AA) intoxication is strongly associated with the development of urothelial abnormalities as observed in AA-intoxicated patients all over the world [18-21] and confirmed in AAN experimental models [22, 23]. In this regard, metabolic activation of AA to species forming DNA adducts is an important step for AA-induced malignant transformation. The major AA-DNA adducts found in AAN animal models and AA-intoxicated patients were identified as 7-(deoxyadenosine-N6-yl) aristolactam I (dA-AAI), 7-(deoxyguanosine-N2-yl) aristolactam I (dGAAI), and 7-(deoxyadenosine-N6-yl) aristolactam II (dAAAII). Among them, dA-AAI is the most persistent AADNA adduct and constitutes a mutagenic lesion leading to excess A: T → T: A transversions [24, 25]. The highest fraction of these transversions occurs in the kidney and the bladder [26]. These specific mutations are retrieved at high frequency in codon 61 of the H-ras protooncogene in tumors induced by AAI in rodent models [27]. In AAN patients, overexpression of p53 protein was observed suggesting a mutation in the tumor suppressor gene, TP53 [28]. In 2004, this mutation was identified as a specific AAG to TAG transversion in codon 139 (Lys → Stop) of exon 5 in the TP53 gene [29]. Interestingly, the same neighboring bases were noticed in the mutated adenine in codon 139 of the TP53 gene and codon 61 of the H-ras gene, suggesting a sequence-specific mechanism during mutation induction [30]. Later, it has been described that A: T → T: A transversions constitute 58% of TP53 sequence changes found in UTUC linked to AA exposure while it represents less than 2% in UTUC patients with no suspected exposure to AA [31]. Moreover, these mutations described in AA-induced UTUC are almost exclusively on the non-transcribed strand which is rather a unique hallmark because in other human cancers, A: T → T: A transversions do not present this pattern [31].

Specifically, mutational hot spots were observed at codons 131 and 179 and the splice acceptor splice site for intron 6 [32]. Mutations in these sites had never been described in UTUC and appear to be uniquely associated with AA exposure. Another recent study reported an unusually high prevalence of G: T transversions in the TP53 binding site in UTUC of non-smoking AAintoxicated women in Belgium (n = 5). The authors proposed these G: T transversions as a complimentary signature mutation for AA intoxication [33].

MicroRNAs interference and UTUC

The rise of omics technology in the last 30 years highlighted the importance of reflecting interactions between environmental factors and genes, to better understand diseases especially diseases with multifactorial etiology such as BEN/UTUC. Recent epigenetics studies show that environmental factors can influence genome function without changing the DNA sequence itself, displaying familial clustering over time [34], and could be implicated in transmitting a "predisposition" over generations [35]. A significant contribution in UTUC development and possible molecular link between the effect of AA intoxication and genetic composition in BEN progression have epigenetic modifications as heritable and adaptable processes at the same time. Therefore, the next step is to introduce a new approach in the field of epigenetics research in BEN/UTUC patients where the same genetic variant combined with the common 'household' AA exposure, results in disease. Among all epigenetic processes (DNA methylation, histone modifications, and miRNA interference), microRNAs are the most powerful regulators of numerous conditions that may critically influence the onset and/or progression of BEN/UTUC disease [36, 37]. MicroRNAs (miRNAs) are a class of highly conserved small RNA molecules, which regulate key biological processes, including cell proliferation, differentiation, development, and metabolism [38]. Aberrantly, expressed miRNAs have been associated with many types of cancers, functioning as regulatory molecules, and acting as oncogenes or tumor suppressors [39, 40]. The human genome contains more than 2000 microRNAs, and it is estimated that 60% of the human protein-coding genes may be regulated by microRNAs, which means they may significantly affect the expression of several proteins [41]. In addition to their regulation by proteins and mRNA, miRNAs can also be controlled by environmental and dietary factors [42,43]. More than half of the miRNA genes are located in cancer-associated genomic regions or fragile sites [44]. Most importantly, different cancer types, stages, or differentiation states have unique miRNA expression profiles, suggesting that miRNAs can function as novel biomarkers for cancer diagnosis [45]. Many studies have investigated the expression of microRNAs in urothelial carcinoma nevertheless

the majority have been performed on the most common urothelial bladder cancer and only a few have included patients with BEN-UTUC [44]. Recently a few studies that investigated microRNA profiles in nephropathies caused by aristolochic acid in humans and rats have been published [46, 47].

Tao et al. compared the expression of microRNA in AAN-UTUC tissues and non-AAN-UTUC tissues in the order to identify unique gene alterations for AAN-UTUC [48]. They have found eight most significantly expressed microRNAs miR-4795-5p ↓, miR-488 ↑, miR-4784 ↓, miR-330 ↓, miR-3916 ↓, miR-4274 ↑, miR-181c ↓, and miR-4434 ↑ in UTUC samples. The study by Meng et al. focuses on the determination of microRNAs that could be used as tissue-specific biomarkers for mutagenicity and carcinogenicity produced by aristolochic acid in rats [48]. They found 19 differentially expressed microRNAs (8 upregulated and 11 downregulated) in the kidney, after oral supplementation with aristolochic acid. Among the most significantly differentially expressed upregulated miRNAs they found miR-21-5p and miR-34a-5p, selecting them as potential biomarkers for carcinogenicity and genotoxicity of aristolochic acid, respectively [49]. Wang et al. worked on rats as animal models to build a microRNA-gene regulatory network to investigate the molecular dynamics induced by aristolochic acid from a systematic perspective [50]. They analyzed the expression data before and after treatment with aristolochic acid to determine the differentially expressed miRNA and obtained 49 significantly differentially expressed miRNAs (32 upregulated and 17 downregulated). The most significantly differentially expressed miRNAs were found to be members of the miR-34, miR-21, miR-224, miR-375, and miR-383 [50].

Popovska Jankovic et al. presented a study of microRNA profiling in UTUC tissues from patients with BEN regions and proposed a panel of 15 differentially expressed microRNA, one downregulated (miR-21) and 14 upregulated (miR-1260a, miR-141-3p, miR-149-5p, miR182-5p, miR-183-5p, miR-197-3p, miR-200c-3p, miR203a-3p, miR-205-5p, miR-205-3p, miR-210-3p, miR224-5p, miR-224-3p, and miR-96-5p) [51]. Another independent study published by Wei et al. who investigated the possibility of using miRNAs as noninvasive markers in the screening or follow-up of UTUC, confirm the expression of the same microRNAs (miRNA-96, miRNA182, miRNA-183, miRNA-141, miRNA-30b, miRNA-21, and miRNA-200c), also overexpressed in UTUC [52]. Results from these studies, strongly suggest that UTUC/BEN patients have unique miRNA expression profiles. Moreover, having in mind the unique signature mutation in UTUC associated with AA exposure and the unique miRNA expression profiles in UTUC we could propose a possible molecular mechanism that could be responsible for the activation of the link between AA intoxication and miRNAs interference in UTUC development in patients with BEN.

Cancer is a group of human diseases with various heterogeneity, which could limit the reproducibility of changes in microRNA expression profiles; even the same tumor lesion may have different gene alterations. For instance, in bladder cancers, low-grade tumors exhibited downregulation of numerous miRNAs, and the most downregulated were miRs - 99a/100, which were demonstrated to target FGFR3 [53]. According to the literature, high-grade bladder cancer often exhibits upregulated levels of miR-21, and miR-21 can target TP53. High-grade bladder cancer is characterized by marked miRNA upregulation [53, 54] whereas low-grade bladder cancer often exhibits miRNA downregulation. AA-UTUC has a distinctive gene alteration pattern, such as AL-DNA adducts, and a unique TP53 mutational spectrum, A: T→T: A, which implies the presence of a distinctive pathway. Following metabolic activation, AA reacts with genomic DNA to form AL-DNA adducts that generate a unique TP53 mutational spectrum in the urothelium (A: T→T: A). It is the most commonly mutated gene in human cancer and is associated with the alteration of cellular bioactivity [55]. Transcription factor p53 protein is a tumor suppressor, p53 protein not only regulates the expression of miRNAs but is also a target of these miRNAs. For example, miR-34, miR -200 family, miR-192 family, miR-107, miR-145, miR15a, and miR-16-1 have been identified to be modulated by p53; while miR-504, miR-33, miR-125b, miR-1285, and miR-380-5p have been reported to directly target p53 [56].

Transversion AAG to TAG in codon 139 (Lys → Stop) of exon 5 in the TP53 gene or A: T→T: A transversion which is present only in UTUC patients intoxicated with AA, has a critical function in the translation, transcription, and activation of p53 protein. In response to AA stimuli, p53 is activated and induces expression of miRNAs, which in turn represses the negative p53 binding regulator (SIRT1) to augment p53 activation [57,58] and cyclin-dependent kinase (CyclinD1) and cyclin protein kinase 6(CDK6) to support cell cycle arrest [59]. SIRT1 deacetylates p53, which decreases the ability of p53 to bind DNA and regulate miRNAs expression [60]. The proposed molecular mechanism represents a unique relationship between AA intoxication, miRNAs expression, and the progression of UTUC in patients with BEN.

DISCUSSION

In 2001, the European Commission on Food Safety suggested aristolochic acid (AA) as the most critical environmental factor for Balkan endemic nephropathy,

likely ingested by contaminated Aristolochia clematitis seeds [60]. The first published data about the etiological mechanism of chronic AA intoxication in BEN was introduced by Ivic in 1967 [61]. He suggested that seeds from Aristolochia, which grow abundantly in wheat fields of endemic areas, were mixed with wheat grain during the harvesting process, and therefore, AA might enter the human food chain through the ingestion of bread prepared from flour derived from contaminated grain. Pavlovic suggested that crops grown in the fields where Aristolochia clematitis grows senesces and decomposes during successive years might accumulate certain amounts of AA from the soil through root uptake and subsequently transfer it to other plant structures [62]. AA was also identified in corn, wheat grain, and soil samples collected from the endemic village of Kutles in Serbia, providing the first direct evidence that food crops and soil are contaminated with AA in Balkan countries and thereby strengthening the intoxication pathway proposed earlier [63]. Observed differences between neighboring villages in the prevalence of BEN could reflect varying levels of exposure based on differences in the microenvironment, agricultural practices, or dietary habits. In affected households, both genetically related and non-related family members are at risk, supporting the argument that household aggregation is more important than heredity [63]. Recently, it is becoming accepted that AA may be responsible for acute and chronic renal failure as the side effects of Aristolochic herbs. However, it is still unclear what happens in the cells after AA intoxication. In this study, the authors using available literature data built possible molecular mechanisms through AA unique TP53 mutational spectrum and miRNA unique expression profiles found only in UTUC to better understand the development and pathology of the patient with BEN.

In conclusion, during the two past decades, animal models for AAN have been developed to investigate underlying molecular and cellular mechanisms involved in AAN pathogenesis. Indeed, a more in-depth understanding of these processes is essential to develop therapeutic strategies aimed to reduce the underestimated burden of this disease. In this regard, our purpose was to build a broad overview of what is currently known about AA, miRNA, and BEN/UTUC.

To achieve this goal, we aimed to summarize the latest literature data available about underlying molecular mechanisms leading to UTUC/BEN development, with a particular emphasis on environmental factors and epigenetics alteration.

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