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Mutational signature of aristolochic acid: Clue to the recognition of a global disease

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ABSTRACT

Mutational signatures associated with specific forms of DNA damage have been identified in several forms of human cancer. Such signatures provide information regarding mechanisms of tumor induction which, in turn, can reduce exposure to carcinogens by shaping public health policy. Using a molecular epidemiologic approach that takes advantage of recent advances in genome sequencing while applying sensitive and specific analytical methods to characterize DNA damage, it has become increasingly possible to establish causative linkages between certain environmental mutagens and disease risk. In this perspective, we use aristolochic acid, a human carcinogen and nephrotoxin found in *Aristolochia* herbs, to illustrate the power and effectiveness of this multidisciplinary approach. The genome-wide mutational signature for this toxin, detected initially in cancers of the upper urinary tract, has subsequently been associated with cancers of the liver and kidney. These findings have significant implications for global public health, especially in China, where millions of individuals have used *Aristolochia* herbal remedies as part of traditional Chinese medicine and, thus, are at risk of developing aristolochic acid nephropathy and/or upper urinary tract carcinomas. The studies reported here set the stage for research into prevention and early detection, both of which will be required to manage a potentially devastating global disease.

1. Introduction

The *Aristolochia* family of herbaceous plants has been used worldwide, for medicinal purposes, for more than 2000 years [1]. Remarkably, the profound toxicities of *Aristolochia* species in humans were recognized only recently [2], when a cluster of approximately 100 otherwise healthy Belgian women developed a rapidly progressive renal disease following ingestion of *Aristolochia fangchi,* which was administered as part of a weight loss regimen. In view of the putative causative agent, the syndrome was designated Chinese herbs nephropathy (CHN). Subsequently, DNA adducts formed with aristolochic acid (AA) were detected in the renal tissues of patients with CHN [3] and a subset of this group developed carcinomas of the upper urinary tract (UTUC) [4]. With continued investigation, researchers noted that CHN exhibited clinical and pathological similarities to another disease known as Balkan endemic nephropathy (BEN) [5].

BEN [6] occurs only in specific farming villages located along the tributaries of the Danube River in Bulgaria, Romania, Serbia, Croatia and Bosnia-Herzegovina. This unusual geographic distribution has remained constant since the 1950s, when BEN was first described as a clinical entity. In the endemic region, *Aristolochia clematitis* grows as a weed in the wheat fields and a hypothesis was proposed to explain the striking similarities between BEN and CHN; namely, that dietary exposure to AA in these farming communities occurs through contamination of wheat grain used to prepare home-baked bread [7]. This hypothesis was confirmed when high levels of aristolactam (AL)-DNA adducts were detected in the renal cortex of patients with BEN; moreover, sequencing of the *TP53* tumor suppressor gene revealed the mutational spectrum associated with AA [8,9]. Taken together, these observations established AA as the long-sought nephrotoxin/carcinogen involved in the etiology of BEN. As a consequence, AA was formally classified as a Group I carcinogen [10] and the terms BEN and CHN were replaced by aristolochic acid nephropathy (AAN) [11].

Abbreviations: AA, aristolochic acid; dAAL-I, 7-(deoxyadenosin-N^6-yl) aristolactam I; dG-AL-I, 7-(deoxyguanosin-N^2-yl) aristolactam I; dA-AL-II, 7-(deoxyadenosin-N^6-yl) aristolactam II; dG-AL-II, 7-(deoxyguanosin-N^2-yl) aristolactam II A deoxyadenosine; T, deoxythymidine.

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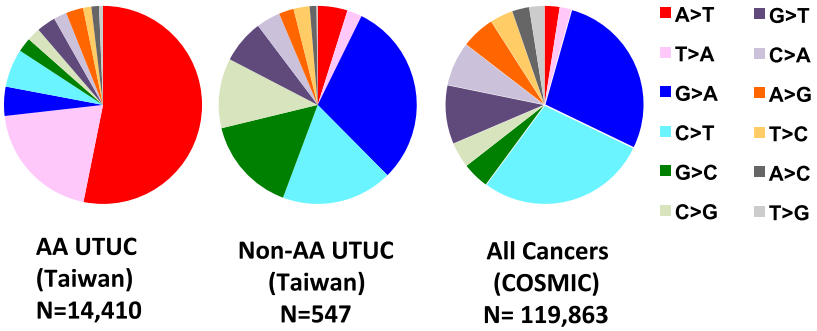

Fig. 1. Mutation spectrum of upper urinary tract carcinomas UTUC. Each pie chart displays single base substitutions established by whole exome sequencing analysis. Data shown were reported by Hoang et al. [20]. Left: a set of 29 tumors from a cohort of Taiwanese patients shown to be exposed to AA by demonstrating the presence of AL-I-DNA adducts in the renal cortex; Middle: a set of seven UTUCs from known smokers among Taiwanese UTUC patients lacking AL-I-DNA adducts in the renal cortex; Right: Mutations from all cancers in the COSMIC database as of 2013. N indicates number of somatic single base mutations in each data set.

To validate the use of dA-AL DNA adducts and of *TP53* mutational spectra as biomarkers for AAN, we undertook a molecular epidemiologic study in Taiwan, where a significant fraction of the population had been prescribed herbal medicines containing AA [12] and the prevalence of upper tract urinary cancer (UTUC) is among the highest in the world. This study showed that more than half of the Taiwanese patients with UTUC had AL-DNA adducts in their renal cortex and that the *TP53* mutational spectrum of their urothelial tumors was essentially identical to those of patients with BEN [13]. We concluded that AA, an intrinsic component of *all Aristolochia* herbal remedies, contributes significantly to the incidence of UTUC in Taiwan [13]. These findings have significant implications for global public health, especially in China, where the use of *Aristolochia* herbs was widespread and millions of people are estimated to be at risk of developing AAN and UTUC [14,15].

It is essential to identify molecular biomarkers that link environmental exposure to specific human cancers. Prior exposure to aristolochic acid can be documented by ^{32}P-postlabelling [16] or by quantitative mass spectrometry analysis [17] of AL-DNA adducts present in fresh or formalin-fixed paraffin-embedded tissues. Using mass spectrometry, the limit of detection of AL-DNA adducts is one adduct per 3×10^9 nucleotides, or just a few adducts per cell [17]. It is known that initiation of carcinogenesis is generally signaled by the presence of mutations in one or more of the involved driver genes. Today, it is possible to identify recurring mutations in cancer genomes and in driver genes as a result of the ability to sequence, at relatively low cost, an entire exome or genome, thus expanding significantly the possibilities of assigning mutational signatures to human carcinogens [18,19]. This task will be greatly facilitated by a fuller understanding of the processes by which specific patterns of mutations are generated; thus, using AA as a model, we selected mutational signatures as the principal focus for this review.

2. Mutational signature of AA in human cancer

2.1. Mutational signature of AA in upper urinary tract carcinoma

Mutations found in tumors that are not present in neighboring non-tumor tissue reflect the sum of all the mutagenic processes active in the history of the cell(s) giving rise to the tumor. Informatics tools have been developed to extract, from genomic sequence data, the "signatures" generated by the mutational processes that predominate in each tumor type. [18,19]. When whole-exome sequences of UTUCs from Taiwan were analyzed, two types of tumor with distinct mutational spectra were revealed [20,21]. In one type, the mutational spectrum resembled that recorded in UTUCs found elsewhere the world. In the second type (Fig. 1), the average number of mutations was greatly elevated, due mainly to a large excess of A-to-T substitutions [20,21]. As had been observed in the *TP53* gene of AA-associated UTUCs [22], the mutated dA residue was located primarily on the non-transcribed strand, most commonly within the trinucleotide 5′-Py_A_G-3′. Most intron splice acceptor sites are 5′AG; as a result, splice acceptor site mutations, specifically A-to-T transversions, are enriched within *TP53* and genome-wide in UTUC patients from Taiwan [22]. UTUCs with the AA-mutational signature possess non-synonomous A-to-T mutations in putative driver genes other than *TP53*, including *MLL2, CREBBP, KDM6A, BRCA2*, and *NRAS*.

Although the specific nature of *TP53* mutations in UTUCs was important in establishing AA as a cause of UTUC, mutation of this gene has certain limitations when used as a biomarker. For example, A-to-T mutations in *TP53* are is present in only ~25% of AA-associated UTUCs. Furthermore, *TP53* is mutated by an A-to-T substitution in ~6% of UTUC worldwide leading to a 25% false positive rate. However, 22 of 26 UTUC with dA-AL-1 DNA adducts contain the genome-wide AA mutational signature [20]. Thus, in future population studies, establishing the presence of the AA-mutational signature genome-wide would be a superior molecular epidemiological tool than determining the mutation status of *TP53*.

2.2. Other human cancers with potential involvement of AA

Since a genome-wide AA-mutational signature was reported for UTUC [20,21], similar signatures were have been detected in other types of cancer, suggesting a wider involvement of AA in human disease (Fig. 2). Bladder transitional cell carcinoma (BC) is similar to UTUC with respect to target tissue and genetic pathways involved. Poon, et al., investigated the contribution of the AA-mutational signature to primary bladder cancer in Taiwan; the AA-signature was present in BC obtained from two Taiwanese patients with known exposure to AA [23].

A deep sequencing study of clear cell renal cell carcinomas (ccRCC) from men and women residing in Britain, Russia, Czech Republic, and Romania revealed an AA-like mutational signature only in Romania (12 of 14 cases [24]). A follow-up study of this group confirmed the presence of dA-AL-I adducts in kidney DNA [25]. RCCs obtained from the AAN endemic region of Croatia also demonstrated the presence of an AA-like mutational signature [26]. Although certain regions of Romania harbor AAN, Romanian patients with the AA-mutational signature were said *not* to have lived in those villages. Thus, the source of AA-exposure in those cases is yet to be determined.

The presence of AL-DNA adducts in the renal cortex, together with the mutational signature clearly establishes the exposure of Romanian patients to AA. However the role of AA-derived adducts in the development of ccRCC is unclear. The *VHL* driver gene is com-

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