Inverted papilloma of the urinary bladder: a molecular genetic appraisal

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Inverted papilloma of urinary bladder is an uncommon urothelial neoplasm. Its relationship to urothelial carcinoma is controversial. Little is known of the genetic abnormalities of inverted papilloma. To better understand its genetics, we analyzed 39 inverted papillomas, including 36 from men and three from women, for loss of heterozygosity (LOH). We examined four polymorphic microsatellite markers located on chromosome 9q32–33(D9S177), chromosome 9p22 (IFNA), chromosome 3p14.2 (D3S1300) and chromosome 17p13.1 (TP53), where genetic alterations occur frequently in urothelial carcinomas. Additionally, the status of inactivation of X-chromosome was examined in three female patients. The frequency of LOH in informative cases was 8% (3 of 37) for D9S177, 10% (4 of 38) for TP53, 8% (3 of 37) for IFNA and 8% (3 of 36) for D3S1300. In the analysis of X-chromosome inactivation, all three cases yielded informative results and one had nonrandom inactivation of X-chromosomes. The monoclonal origin demonstrated in the study of X-chromosome inactivation indicates the clonal process of inverted papilloma; however, the low incidence of LOH supports the view that inverted papilloma in urinary bladder is a benign neoplasm with molecular genetic abnormalities different from those of urothelial carcinoma.

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Inverted papilloma urinary bladder is an uncommon urothelial neoplasm of which makes up <1% of all urothelial neoplasms.¹ While it is generally regarded as a benign neoplasm, conflicting data on recurrence rate and association with synchronous and metachronous urothelial carcinoma have left uncertainties concerning the relationship of inverted papilloma with urothelial carcinoma.^{2–10}

Oncogene activation and tumor suppressor gene inactivation are important mechanisms in the genesis, propagation and spread of urothelial carcinoma.^{11–19} However, little or nothing is known of the molecular genetic events in the development of inverted papilloma of the urinary bladder. This

study was conducted to investigate genetic alterations in inverted papilloma by evaluating the prevalence of losses of heterozygosity (LOH), which are common in urothelial carcinoma and to analyze the X-chromosome inactivation status in inverted papilloma.

Materials and methods

Patients

Inverted papillomas in the urinary bladder were retrieved from the surgical pathology archives of the Departments of Pathology of Indiana University (Indianapolis, IN, USA), Cordoba University (Cordoba, Spain), Chang Gung Memorial Hospital (Kaohsiung, Taiwan) and Singapore General Hospital (Singapore) between 1986 and 2004. All slides were retrospectively reviewed and diagnosed according to the accepted criteria.¹ Patients with insufficient tissue specimens for genetic analysis were excluded. Thirty-nine cases, including 36 from

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men and three from women, fulfilled the requirement were selected into this study.

Tumor Samples and Microdissection

Histological sections were prepared from formalin-fixed, paraffin-embedded blocks and stained with hematoxylin and eosin for histological examination and microdissection. Genomic DNA was prepared from tumor cells of inverted papilloma, microdis-

sected by the PixCell II laser capture microdissection system (Arcturus, Mountain View, CA, USA), as previously described (Figure 1).^{20–22} Approximately 400–600 cells were microdissected from 5- μ m histologic sections. Normal tissues microdissected from the same specimen were used as a control sample for each patient.

Detection of LOH

The following four oligonucleotide primer pairs for microsatellite DNA markers were chosen on the basis of their frequent alteration in urothelial carcinoma and papillary urothelial neoplasm of low malignant potential: D3S1300 (chromosome 3p14.2), IFNA (chromosome 9p22), D9S177 (chromosome 9q32–33) and TP53 (chromosome 17p13.1).^{11,13,14,18,21,23,24} Polymerase chain reaction (PCR) and gel electrophoresis were performed for all 39 cases, as previously described.^{20,25,26} LOH was determined by a combination of direct visualization and scanning densitometry of the autoradiograms with a phosphorimager (Molecular Dynamics Inc., Sunnyvale, CA, USA). The criterion for allelic loss was complete or near complete absence of one allele in tumor DNA as previously described.^{25–27} PCRs for each polymorphic microsatellite marker were repeated at least twice from the same DNA preparations, and the same results were obtained. The results were reported as noninformative when two distinct bands could not be distinguished in control DNA after PCR amplification.

Detection of X-Chromosome Inactivation

X-chromosome analysis was performed in the three tumors from women. The microdissected tissues were placed in 50 μ l of buffer (10 mM Tris, 1 mM EDTA, 1% Tween-20 and 5 mg/ml proteinase K (pH 8.3)) and incubated overnight at 37°C for DNA extraction.^{26,28} Eight microliter aliquots of the DNA extract were digested overnight with 1 U of *Hha*I restriction endonuclease (New England Biolabs Inc., Beverly, MA, USA) in a total volume of 10 μ l. Control reactions for each sample were incubated in the digestion buffer without *Hha*I endonuclease. Primers used in this reaction were: AR-sense: 5'TCC AGA ATC TGT TCC AGA GCG TGC3' and AR-antisense: 5'GCT GTG AAG GTT GCT GTT CCT CAT3'. Polymerase chain reaction and gel electrophoresis were performed as described.^{26,28}

The clonality of the samples was evaluated on the basis of a polymorphism of the X-linked *human androgen receptor* gene (*HUMARA*) locus.^{26,28–30} This technique is dependent on digestion of DNA with the methylation-sensitive restriction enzyme *Hha*I, PCR amplification of the *HUMARA* locus and the detection of methylation at this locus. With this method, only the methylated *HUMARA* allele is amplified by PCR. The random inactive status of an

Figure 1 Laser capture microdissection of inverted papilloma in urinary bladder. (a) Tumor specimen before microdissection. (b) Tumor specimen after microdissection. (c) Laser-captured tumor cells.

X chromosome is established in all female somatic cells early in embryogenesis.³¹ Normal female tissues should be a cellular mosaic, with an equal distribution of cells containing maternal or paternal-derived inactivated X chromosomes. Nonrandom X chromosome inactivation indicates a clonal process.²⁹

The cases were considered informative if the control sample displayed two alleles after PCR amplification without *HhaI* digestion. Nonrandom inactivation of the X chromosomes was defined as a complete or nearly complete absence of one or the other allele after *HhaI* digestion, indicating predominance of one androgen receptor allele.

Results

We analyzed LOH in 39 inverted papillomas, including 36 from men and three from women, as well as X-chromosome inactivation status in the three tumors from women (Figure 2). In the study of LOH, the pattern was informative for 37 tumors (95%) at the D9S199 locus, 38 patients (97%) at the

TP53 locus, 37 patients (95%) at the IFNA locus and 36 patients (92%) at the D3S1300 locus. The frequency of LOH in informative cases was 8% (3 of 37) for D9S177, 10% (4 of 38) for TP53, 8% (3 of 37) for IFNA and 8% (3 of 36) for D3S1300. Among the tumors demonstrating LOH, one of them (case 2) revealed LOH in two loci (TP53 and D3S1300) and the remaining tumors showed loss of allele in only one locus. None of normal tissues showed LOH in this study.

The tumors from the three women were examined by X-chromosome inactivation analysis. All three cases yielded informative results. Nonrandom inactivation of X-chromosomes was identified in one tumor (case 39), while random inactivation of X-chromosomes was identified in the other two (case 19 and 25).

Discussion

In our study of inverted urothelial papilloma analyzed by LOH and X-chromosome inactivation, both the low incidence of LOH and nonrandom.

Figure 2 Representative results of LOH (a) and X-chromosome inactivation analysis (b) in inverted papilloma of the urinary bladder. In panel a, DNA was prepared from normal and tumor tissue, amplified by polymerase chain reaction using polymorphic markers D3S1300, IFNA, D9S177 and TP53 and separated by gel electrophoresis. In panel b, nonrandom inactivation of X-chromosome was identified in case 39. Arrows: allelic bands, N: normal control tissue, T: tumor tissue of inverted papilloma, -: without *HhaI* endonuclease digestion, +: with *HhaI* endonuclease digestion.

Table 1 Comparison of LOH between inverted papilloma, papillary urothelial neoplasm of low malignant potential and urothelial carcinoma

Reference	Specimen	Frequency of LOH (%) chromosome region			
		D9S177 9q32–33	IFNA 9p22	TP53 17p13.1	D3S1300 3p14.2
Current study	Inverted papilloma	8	8	10	8
Cheng <i>et al</i> ²¹	PUNLMP	41	32	29	44
Baud <i>et al</i> ¹¹	Urothelial carcinoma	74			
Paterson <i>et al</i> ²³	Urothelial carcinoma	67		47	
Keen <i>et al</i> ¹³	Urothelial carcinoma		60		
Uchida <i>et al</i> ²⁴	Urothelial carcinoma		35	39	
Primdahl <i>et al</i> ¹⁸	Urothelial carcinoma		35	41	
Louhelainen <i>et al</i> ¹⁴	Urothelial carcinoma				80

LOH: loss of heterozygosity; PUNLMP: papillary urothelial neoplasm of low malignant potential.

X-chromosome inactivation support the notion that inverted papilloma in urinary bladder is a benign urothelial neoplasm, lacking the molecular genetic abnormalities typical of urothelial carcinomas.

Cheng *et al*³² reviewed the English-language literature, including 302 inverted papillomas in the lower urinary tract, and found that the recurrence rate was 4% and the incidences of associated synchronous urothelial carcinoma and development of subsequent urothelial carcinoma was 6 and 3%, respectively. Overaccumulation of p53 protein, overexpression of HER-2/neu and increased proliferative index have been identified in inverted papillomas, raising the possibility that it might have the potential for future malignant transformation.^{4,9} Amin *et al*³³ reported that urothelial carcinoma with an inverted growth pattern may have features resembling inverted papilloma and they considered reports of the ‘coexistence’ of inverted papilloma and urothelial carcinoma to be examples of urothelial carcinoma with inverted architecture. In a study of 11 inverted urothelial papillomas with atypia by Broussard *et al*,³ they concluded that those cases reported as ‘inverted papilloma with malignant features’ were best classified as either urothelial carcinoma with inverted pattern or inverted papilloma with atypia.

Recent advances in molecular genetics have shown that urothelial carcinoma arises from the accumulation of genetic alterations in oncogenes and tumor suppressor genes.^{15–17,19} Herein we studied the status of LOH in inverted papilloma using four microsatellite markers, which commonly are altered in urothelial carcinomas.^{11,13,14,18,21,23,24} Our results are compared with those of the previous studies in Table 1. The incidence of LOH in inverted papilloma is low (8–10%) and contrasts with the high frequency of LOH (29–80%) in urothelial carcinomas and papillary urothelial neoplasms of low malignant potential.^{11,13,14,18,21,23,24} The low incidence of LOH in inverted papilloma is similar to the one in normal urothelium, in which Junker *et al*³⁴ reported that 6% cases with benign prostatic

hyperplasia demonstrated microsatellite alteration on chromosome 9 in their normal urothelial tissue. The opposite expression of LOH between inverted papilloma and urothelial carcinoma may suggest that inverted papilloma usually does not harbor the key genetic changes common in urothelial carcinoma and both entities probably have their own distinct pathogenetic mechanism.

In an investigation to examine LOH patterns in papillary urothelial hyperplasia and urothelial papilloma, Chow *et al*³⁵ found eight of 15 (53%) cases of papillary urothelial hyperplasia were clonal, demonstrating LOH of at least one marker. Additionally, an identical pattern of LOH between papillary urothelial hyperplasia and subsequently recurrent urothelial carcinoma was identified in one patient. In contrast, none of four papillomas revealed any genetic alteration among the LOH markers tested. Their molecular data suggested that papillary urothelial hyperplasia may be a precursor lesion of urothelial carcinoma, but there was no genetic evidence to support the precursor nature of papilloma. Neither urothelial papilloma nor urothelial inverted papilloma shares the common genetic alterations frequently seen in malignant urothelial neoplasm, which may reflect their favorable prognoses and benign clinical courses; suggesting distinct pathogenetic pathways. However, due to the limited case number (4) of urothelial papilloma included in the previous study,³⁵ a further study is warrant to arrive on a conclusion of genetic nature of urothelial papilloma, especially considering the fact that a small portion of urothelial papilloma may recur³⁶ or even progress to develop urothelial carcinoma.³⁷

The most consistently informative marker of the clonal composition of neoplasm in female subjects is the nonrandom pattern of X-chromosome inactivation.^{26,29} Polyclonal proliferative lesions in female patients are composed of cells with different origins and randomly inactivated X-chromosomes will be identified within them, while neoplasms arising from a single clone are composed of cells in which

the same X-chromosome is inactivated.^{26,29,38,39} In our study, one inverted papilloma showed nonrandom inactivation of X-chromosomes, suggesting that inverted papillomas arise from a single progenitor cell and supporting the clonal process of inverted papilloma.

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