ORIGINAL ARTICLE

DNA repair genes and prognosis in sporadic forms of urothelial carcinoma of the upper urinary tract

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DNA repair genes;
Microsatellite instability;
Urothelial carcinoma;
Upper urinary tract;
Prognosis

Abstract

Introduction: Lynch syndrome or hereditary nonpolyposis colorectal cancer is caused by mutations in DNA repair genes, known as mismatch repair (MMR) genes, and is associated with microsatellite instability. Urothelial carcinoma of the renal pelvis is also associated with this syndrome. These genetic abnormalities have been described in sporadic forms of upper tract urothelial carcinoma (UTUC).

Material and method: This was a descriptive study and survival analysis of a series of 80 patients with sporadic UTUC with no metastases at diagnosis (N0/Nx M0) treated exclusively with nephroureterectomy. We evaluated the expression of MMR genes (hMLH1, hPMS2, hMSH2 and hMSH6) in sections performed with tissue microarray (TMA) and their association with clinical-pathological parameters. We analyzed the prognostic value of the loss of expression of these genes in UTUC.

Results: We detected no loss of MSH2 or of MSH6, but there was a loss of MLH1 in 11 cases (13.8%) and of PMS2 in 21 cases (26.3%). The expression of hMLH1 and hPMS2 were strongly associated (P < .0001), and this phenotype expression entails significant clinical implications. The loss of MLH1 was associated with a low grade (P = .02). Loss of PMS2 was associated with a lower stage (P = .05), a pushing pattern with no invasive edges (P = .008) and less angiogenesis (P = .008). The inactivation of hPMS2 or hMLH1 is an independent protective factor (HR, 0.309) and, along with the histologic grade (HR, 5.561), defines the patients' prognosis.

Conclusion: In our experience, the inactivation of hPMS2 or hMLH1 is an independent marker of good prognosis and occurs in a quarter of sporadic UTUC cases. The immunohistochemical study of these patients can be used to assess the screening of hidden forms of Lynch syndrome.

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PALABRAS CLAVE
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Inestabilidad de microsatélites;
Carcinoma urotelial;
Tracto urinario superior;
Pronóstico

Genes reparadores del ADN y pronóstico en formas esporádicas de carcinoma urotelial del tracto urinario superior

Resumen
Introducción: El síndrome de Lynch o cáncer colorrectal hereditario no polipósico se debe a mutaciones en los genes de reparación de ADN, conocidos como mismatch repair (MMR), y se asociarían a inestabilidad de microsatélites. El cáncer urotelial de la pelvis renal también se asocia a este síndrome, e incluso estas anomalías genéticas se han descrito en formas esporádicas de carcinoma urotelial del tracto superior (CU-TUS).

Material y método: Estudio descriptivo y análisis de supervivencia en una serie de 80 pacientes con CU-TUS esporádico sin metástasis al diagnóstico (N0/Nx M0) tratados mediante nefroureterectomía exclusivamente. Se evalúa la expresión de genes MMR (hMLH1, hPMS2, hMSH2 y hMSH6) en secciones realizadas con microarray de tejido (TMA) y su asociación con parámetros clínico-patológicos. Se analiza el valor pronóstico de la pérdida de expresión de estos genes en CU-TUS.

Resultados: No se detectó pérdida de MSH2 ni de MSH6, pero se evidenció pérdida de MLH1 en 11 casos (13,8%) y de PMS2 en 21 (26,3%). La expresión de hMLH1 y hPMS2 se encuentra fuertemente asociada (p < 0,0001) y este fenotipo de expresión conlleva importantes implicaciones clínicas. Pérdida de MLH1 se asocia con bajo grado (p = 0,02). Pérdida de PMS2 se asocia con menor estadio (p = 0,05), patrón empujante sin borde invasivo (p = 0,008) y menos angiogénesis (p = 0,008). La inactivación de hPMS2 o hMLH1 es un factor protector independiente (HR 0,309) y junto con el grado histológico (HR 5,561) define el pronóstico de estos pacientes.

Conclusión: En nuestra experiencia la inactivación de hPMS2 o hMLH1 es un marcador independiente de buen pronóstico y sucede en la cuarta parte de los CU-TUS esporádicos. El estudio ILH de estos pacientes puede emplearse para valorar el cribado de formas ocultas de síndrome de Lynch.

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Introduction

Hereditary non-polyposis colorectal cancer (HNPCC), also known as Lynch syndrome, is a family entity with an autosomal inheritance pattern conditioning a high probability of developing a malignant disease of the large intestine throughout life. This disease is responsible for 1–5% of colon and rectum carcinomas. It typically occurs at an early age, about two decades before sporadic colorectal carcinoma. It is also associated with neoplasms in extracolonic location, specifically endometrial and ovarian cancer, gastric and small bowel cancer, cancer of the hepatobiliary system, skin cancer (Muir–Torre syndrome), and cancer of the central nervous system and the genitourinary tract. At a urological level, urothelial carcinoma of the upper urinary tract (UC-UUT) has been well described, these individuals with HNPCC showing a 14-fold increased risk of developing this neoplasm than the general population.

The genetic instability inherent to cancer may occur either as a result of a high frequency of chromosomal aberrations or mutations due to, at least partly, defects in genes of the DNA repair system, known as mismatch repair (MMR), which are associated with microsatellite instabilities (MSI) (Fig. 1). The MMR system fulfills its function by providing normal cells a high level of protection against mutations in DNA replication, and is composed of a family of proteins, encoded for by the genes hMSH2, hMSH3, hMSH6, hMLH1, hPMS1 and hPMS2, the inactivation of which plays a prominent role in the development of cancer caused by a high mutation rate. Neoplasms occurring in the context of HNPCC are characterized by showing mutations in the germ-line of one or several of these genes.

Microsatellites are short, polymorphic segments with tandem repetitions which appear dispersed throughout the genome. Microsatellite instability may be evidenced through

Figure 1 Diagram showing complex MRI during the repair of one mismatch in DNA.
PCR and sequencing procedures by detecting repetition markers of three mononucleotides such as BAT25 (4q12), BAT26 (2p16) or BAT40 (1p13.1), and by checking heterozygosity loss with dinucleotide markers in D9S171 (9p21) and D5S346 (5q22) or also by checking if there is abnormal methylation of the hMLH1 promoter.\textsuperscript{13,14} From a practical viewpoint, loss of immunohistochemical (IHC) staining for at least one of the MMR proteins implies a much more accessible technique for checking the presence of a phenotype characteristic of microsatellite instability.\textsuperscript{15,16}

Numerous studies show evidence of high microsatellite instability in urothelial carcinoma, either associated with HNPCC\textsuperscript{15-19} or sporadic; the latter not just in the upper urinary tract,\textsuperscript{3,12,15,19-21} but also in the bladder.\textsuperscript{14,22-24} It is not well defined whether the existence of a phenotype of microsatellite instability in sporadic forms of UC-UUT entails a different prognosis. In this regard, the present study aims at analyzing the clinicopathological factors associated with such a phenotype and its involvement in prognosis.

**Materials and methods**

**Patients and samples**

A prospective study carried out on a series of patients with a histological diagnosis of UC of the renal pelvis with no metastatic involvement (NO/Nx MO) treated with radical nephroureterectomy between January 2000 and December 2008. All records were included in a database with the approval of the institutional review board. Surgery was performed through an open or laparoscopic approach, and lymphadenectomy was performed according to the surgeon’s preferences, without using a predefined template. Patients were followed-up every 3-4 months for the first year, then every 6 months for another 4 years and subsequently on an annual basis in order to rule out recurrence in the retroperitoneum and/or distant metastasis. Follow-up of the urothelial tract was also carried out, although the occurrence of recurrences at the bladder level was not analyzed in this study. In all patients TCC was the primary onset of the disease, those patients with a history of previous bladder cancer not being included. Patients were followed-up until their death from the disease or until data were censored. A single pathologist (J.I.L) assessed all the samples identifying the histological type, grade (WHO 1973 and WHO/USIP 2004), tumor stage (AJCC/TNM 2010), invasion pattern by defining the invasive edge, vascular-lymphatic permeation, infiltration of the renal parenchyma, tumor necrosis and neoformed vessels.

**Tissue microarray construction**

Tissue microarray (TMA) was constructed using tissue samples embedded in paraffin and fixed in formalin from 80 patients. The original preparations were revised and those containing abundant tumor tissue and representative of the case were marked with colored ink. For each case, three tumor cylinders were transferred (0.6 cm in diameter) from the selected areas to the recipient block. 5-μm sections were cut from the TMA blocks and stained with hematoxylin–eosin, verifying the proper construction of the blocks and that all cases were representative ones. The definitive analysis included 240 tumor cylinders which were fully examined.

**Immunohistochemical staining**

Preparations were deparaffinized, rehydrated and an IHC study was performed for MLH1, PMS2, MSH2 and MSH6 using the streptavidin-biotin-peroxidase method modified with diaminobenzidine as a chromagen. The antibody panel used included MutL Protein Homolog 1 (monoclonal MLH1, clone ES05; DAKO, Glostrup, Denmark), Postmeiotic Segregation Increased 2 (monoclonal PMS2, clone EP51; DAKO, Glostrup, Denmark) and MutS Protyein Homolog 6 (monoclonal MSH6, clone EP49; DAKO, Glostrup, Denmark). Positive controls were carried out with colon cancer samples which overexpressed antibodies. Negative controls were also performed without antibody application. All sections were revised by the same pathologist (Fig. 2).

**Statistical analysis**

The presence or absence of nuclear staining protein in urothelial carcinoma was recorded in each case, with loss of IHC expression being defined as the absence of immunostaining in the tumor tissue with persistence of staining in the healthy stroma or in the non-neoplastic urothelium. The frequency of expression loss was studied for each marker. The association of the immunohistochemical phenotype with other variables (Chi-square, Fisher’s and Cochran–Armitage tests) was analyzed and a survival study for cancer-specific mortality was performed by calculating Kaplan–Meier curves, and performing the comparisons between those curves (log-rank test). Likewise, Cox’s proportional hazards models were applied in order to determine possible prognostic factors, both univariately and multivariately.

**Results**

The series analyzed consisted of 80 patients with sporadic UC-UUT. The clinicopathological characteristics of the series were described in a previous study.\textsuperscript{23} With regard to the location of lesions in the upper urinary tract, 63 (78.8%) occurred in the renal pelvis and 25 (31.3%) in the pelvis and ureter. According to the TNM 2009 classification, 44 (55%) tumors were classified as pT1, 9 (11.3%) as pT2, 23 (28.7%) pT3, and 4 (5%) as pT4. In 65 cases (81.3%) the infiltrative growth pattern showed a pushing edge, whereas in 15 (8.7%) cases it was invasive. In 4 of the 18 cases where lymphadenectomy was performed lymph-node involvement was confirmed. Non-transitional histological differentiation was observed in 7 (8.7%) cases, renal parenchymal invasion in 22 (27.5%), vasculolymphatic invasion in 15 (18.7%) and tumor necrosis in 15 (18.7%). With regard to grade, according to the WHO 1973 classification, 8 (10%) were grade I, 55 (68.7%) grade 2 and 17 (21.3%) grade 3. According to the 2004 WHO/USIP classification, 42 (52.5%) were low grade and 38 (47.5%) were high grade.
DNA repair genes and renal pelvis carcinoma

The mean follow-up of patients was 47.8 ± 38.8 (4–172) months. Throughout this period, death from the disease was recorded in 28.7% of the series. Cancer-specific survival was 87.2% (CI 95%: 0.77–0.92) at 12 months, 73.4% (CI 95%: 0.61–0.82) at 36 months, 71.4% (CI 95%: 0.58–0.80) at 60 months and 62.2% (CI 95%: 0.45–0.75) at 120 months (Fig. 3). Patients with high-grade tumors showed poorer survival than those with low-grade tumors (p < 0.0001) (Fig. 4). Similarly, those with infiltration of the muscular layer of the renal pelvis wall in the nephroureterectomy specimen, or even at a deeper level (pT2–pT4), showed poorer cancer-specific survival than those who did not show infiltration of the muscular layer (pTa–pT1) (p = 0.004) (Fig. 5).

All patients (100%) expressed MSH2 and MSH6; 69 (86.3%) showed positive nuclear staining for MLH1 and 59 (73.7%) for PMS2. The expressions of MLH1 and PMS2 were strongly associated (Cramer’s V, p = 0.58; Fisher’s test, p < 0.00001). Twenty-two cases (27.5%) showed a phenotype of microsatellite instability with loss either of MLH1 or PMS2.

The absence of MLH1 was associated with low tumor grade (p = 0.02, WHO 1973; p = 0.036, WHO/USIP 2004). Similarly, although without reaching statistical significance, pT category (p = 0.09) and the presence of angiogenesis (p = 0.08) was lower in these cases. MLH1 expression was not associated with age, gender, renal parenchymal invasion, vascular-lymphatic permeation or tumor necrosis (Table 1). Loss of PMS2 was associated with lesions of a lower pT category (pTa–pT1) (p = 0.05), a non-invasive pushing growth pattern (p = 0.008) and absence of neoformed vessels in tumor tissue (p = 0.008). PMS2 expression was not associated with age, gender, tumor grade, renal parenchymal invasion, vascular-lymphatic permeation or tumor necrosis (Table 1).

Loss of MLH1 or PMS2 (one or the other) is in this series a favorable prognostic factor for cancer-specific survival (log-rank, p = 0.027) (Fig. 6). In fact, 100% of these patients survive the first year, 90.6% (0.67–0.97) the first three years and 85% (0.60–0.95) the first 5 and 10 years, whereas in

Figure 2  IHC staining with conserved expression of MLH1 (A) and PMS2 (B).

Figure 3  Curves of cancer-specific survival for the whole series.
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Discussion

Surely due to the serious difficulties to make an early diagnosis, UC-UUT is a neoplasm which often shows an aggressive clinical behavior. This poor survival is particularly unfavorable in patients with advanced and high-grade tumors, which have an elevated risk for vascular invasion or hidden metastatic involvement. For decades, different studies have been made to identify clinical, histopathological and molecular prognostic factors, although the relative rarity of this disease make prospective studies scarce.

There is great heterogeneity among the different tumor stages of the AJCC/UICC classification. As if this were not enough, the impact of lymphadenectomy on these patients is also discussed. Besides, in the sporadic forms of UC-UUT several specific etiologic factors are recognized, such...
as Balkan nephropathy, nephropathy due to analgesic abuse or the consumption of Chinese herbs; but we do know that there are other forms with inheritance patterns. UC-UUT occurs in approximately 5% of patients suffering from HNPCC, so it represents the third neoplasm in order of frequency in this hereditary syndrome, surpassed by colorectal (63%) and endometrial cancer (9%).

![Survival estimates of the product-limit with number of subjects at risk](Image)

**Figure 6** Survival according to expression of MMR genes (hMLH1, hPMS2).

### Table 1  Relationship between expression of hMLH1 and hPMS2 and different clinicopathological variables.

<table>
<thead>
<tr>
<th>Variables</th>
<th>hMLH1</th>
<th>hPMS2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>p = 0.63</td>
<td>p = 0.067</td>
</tr>
<tr>
<td>Sex</td>
<td>p = 0.67</td>
<td>p = 0.75</td>
</tr>
<tr>
<td>Ureteral involvement</td>
<td>p = 0.09</td>
<td>p = 0.78</td>
</tr>
<tr>
<td>Histological variety</td>
<td>p = 1.0</td>
<td>p = 1.0</td>
</tr>
<tr>
<td>Way of involvement</td>
<td>p = 0.11</td>
<td>p = 0.008</td>
</tr>
<tr>
<td>Grade WHO 1973</td>
<td>p = 0.02</td>
<td>p = 0.07</td>
</tr>
<tr>
<td>Grade WHO/USIP 2004</td>
<td>p = 0.03</td>
<td>p = 0.61</td>
</tr>
<tr>
<td>pT AJCC/TNM 2010</td>
<td>p = 0.09</td>
<td>p = 0.05</td>
</tr>
<tr>
<td>Renal involvement</td>
<td>p = 0.27</td>
<td>p = 0.4</td>
</tr>
<tr>
<td>Vascular involvement</td>
<td>p = 0.37</td>
<td>p = 0.33</td>
</tr>
<tr>
<td>Necrosis</td>
<td>p = 0.67</td>
<td>p = 0.33</td>
</tr>
<tr>
<td>Newly formed vessels</td>
<td>p = 0.08</td>
<td>p = 0.008</td>
</tr>
</tbody>
</table>

a Histological variety that distinguishes pure urothelial carcinoma (TCC) and TCC with other forms of differentiation (squamous, glandular, small cell).

b Way of involvement that distinguishes between pushing and infiltrating pattern.

c Newly formed vessels in the tumor thickness shown by CD31 immunostaining.

The incidence of UC-UUT in HNPCC is higher than the sporadic form of the disease and the mean age of presentation is also an earlier age.

The HNPCC syndrome develops neoplasms due to the accumulation of repetitive short polymorphic sequences dispersed through the human genome as a result of the alteration of the genes responsible for (MMR system) fixing these matching errors during DNA replication (Fig. 1). This type of characteristic alterations may also be present in some sporadic carcinomas, including UC-UUT. The main DNA-repair genes (MMR) are hMLH1, hMSH2, hMSH6, hMSH3, hPMS2, hPMS1 and hMLH3. The loss of function of these proteins requires the inactivation of the two alleles as a consequence of germ-line mutation in HNPCC syndrome, by hypermethylation of the MLH1 promoter in the sporadic cases or by deletion. If MLH1 or MSH2 are definitively altered, a mutation storm occurs due to the lack of replication error correction, which multiplies the spontaneous mutation rate of normal cells by 100 and even by 1000 and conditions microsatellite instability.

As in the case of other forms of colon cancer which also show microsatellite instability, better response to chemotherapeutic regimens was defined with sporadic UC-UUT showing this same phenotype. We even know that UC-UUTs associated with HNPCC may be a good candidate for considering endourological treatment and watchful follow-up, avoiding nephroureterectomy depending on its lower clinical aggressiveness. A non-invasive method based on the detection of heterozygosity loss and microsatellite instability in cells exfoliated in urine may result in establishing a differential molecular profile for this kind of urothelial lesions. Even changes in the expression of MMR genes in

### Table 2  Independent predictors of cancer-specific survival in the multivariate analysis.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>β coefficient</th>
<th>SE</th>
<th>Chi-square</th>
<th>P-value</th>
<th>HR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>High histological grade</td>
<td>1.716</td>
<td>0.512</td>
<td>11.212</td>
<td>0.0008</td>
<td>5.561</td>
<td>2.037–15.180</td>
</tr>
<tr>
<td>Loss of hMLH1 or hPMS2</td>
<td>-1.175</td>
<td>0.580</td>
<td>4.098</td>
<td>0.043</td>
<td>0.309</td>
<td>0.099–0.963</td>
</tr>
</tbody>
</table>
tissue, definable through IHC, also reflect microsatellite instability; although this may not be the most appropriate method to conduct screening of functional alterations in hMLH1. It seems very likely that, as in the case of colon cancer, most MMR-deficient UC-UUTs are sporadic and that only a portion of these individuals are subject to inherited predisposition. But, on the other hand, this issue is more complicated if we accept that a proportion of individuals suffering from Lynch syndrome show microsatellite instability but are immunoreactive to IHC. Since 1998, we have known that some sporadic forms of UC-UUT are associated with microsatellite instability, and that this finding is more frequent in young women, especially in forms affecting the ureter, and in urothelial tumors with an inverted growth pattern. According to our experience with sporadic forms of UC-UUT, loss of hMLH1 and hPMS2 expression occurs more frequently in tumors showing a pushing and non-invasive involvement pattern of the subepithelial connective, but we did not appreciate any association at a younger age or in the case of females. They are neoplasms of a lower grade and with less infiltration capacity, which gives this kind of patients a better prognosis than the sporadic forms that do not show microsatellite instability.

Another study revealed that sporadic forms of UC-UUT with absent hMLH1 and hMSH2 expression tend to present themselves as lesions at an early stage. These data support the possibility that forms with MMR deficiency due to genetic alterations imply significant biological and clinical consequences. In fact, in our experience, hPMS2 or hMLH1 inactivation is an independent marker for good prognosis and occurs in a quarter of sporadic UC-UUTs. This fact involves two great conclusions. Firstly, if an IHC study had been performed on a biopsy obtained through ureteroscopy before nephroureterectomy, it would have been possible to consider non-radical primary endourological treatment in this type of patients with a better prognosis. On the other hand, though no less important, it is possible to consider the suitability of screening HNPPC and other associated neoplasms in patients with an IHC pattern suggestive of MMR expression loss to detect newly diagnosed forms of Lynch syndrome. The relevance of obtaining a complete history of cancer focused on both a personal and family history in patients with UC-UUT seems evident. Only in this way will we be able to identify patients with HNPPC. Thus, these patients and their families will be able to benefit from follow-up and proper management programs.

Conflict of interest
The authors declare that they have no conflict of interest.

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