ELECTRONIC LETTER

Microsatellite instability as indicator of *MSH2* gene mutation in patients with upper urinary tract transitional cell carcinoma

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Upper urinary tract transitional cell carcinoma (UUTTCC) accounts for 5% of all urothelial carcinomas.[1] Hereditary non-polyposis colorectal cancer (HNPCC) is an autosomal dominant syndrome predisposing to colorectal cancer that accounts for about 5% of all colorectal cancers.[2] It is revealed by colorectal cancer (63%) or extracolonic cancers, most often of the endometrium (9%) or ovary, but sometimes of the upper urinary tract (5%), small bowel, stomach, or hepatobiliary tract.[3–5] Patients with hereditary non-polyposis colorectal cancer have a genetic risk of developing these cancers.[3 6]

This study concerns patients with upper urinary tract transitional cell carcinoma who did not meet the international clinical criteria (Amsterdam criteria)[5] for hereditary non-polyposis colorectal cancer. The question that arises is whether the biochemical and molecular biology tests used to screen for hereditary non-polyposis colorectal cancer might not help detect hereditary disease among these cases of upper urinary tract transitional cell carcinoma.[7]

The tests used in suspected cases of hereditary non-polyposis colorectal cancer are:

- *Microsatellite instability detection*. Microsatellite instability is an indication that there have probably been mutations in genes encoding proteins involved in DNA repair. It is an indicator of the clonal expansion of neoplasms and was first identified in the tumours of patients with hereditary non-polyposis colorectal cancer; high microsatellite instability levels are nearly always present in these tumours.[2 8–11] Microsatellite instability is found in almost 40% (39–46) of cases of upper urinary tract transitional cell carcinoma (high and low levels).[12 13]
- *Immunohistochemistry*. Immunohistochemistry is used to evaluate loss of protein expression. Hereditary non-polyposis colorectal cancer is caused by germline mutations affecting one or several mismatch repair genes—that is, genes *hMSH2* (60% of the time), *hMHL1* (30%) and, more rarely, *hMSH3, hPSM2*, and *hMSH6*.[2 14] Colon cancers with high microsatellite instability levels in hereditary non-polyposis colorectal cancer patients no longer express the protein products of the *hMLH1* and *hMLH2* genes.[9 15]
- *Genetic analysis*. The coding exons of the genes are amplified by polymerase chain reaction (PCR) and analysed by single strand conformation polymorphism (SSCP) and sequencing to detect germline mutations.

Screening strategies based on the above tests have been investigated recently in other clinical situations.[16 17] The aim of this study was twofold: (*a*) to establish the benefits of routine screening for microsatellite instability in patients with upper urinary tract transitional cell carcinoma who do not meet the Amsterdam criteria, and (*b*) to establish criteria for the selection of patients with upper urinary tract transitional cell carcinoma in whom immunohistochemistry and possibly genetic testing should be performed. A similar

Key points

- Hereditary non-polyposis colorectal cancer (HNPCC) is an autosomal dominant syndrome predisposing to colorectal cancer which is revealed by colorectal cancer (63%) or extracolonic cancers, most often of the endometrium (9%) or ovary, but sometimes of the upper urinary tract (5%).
- Our aim was: (*a*) to establish the benefits of routine screening for microsatellite instability in patients with upper urinary tract transitional cell carcinoma (UUTTCC) who did not meet the Amsterdam criteria for hereditary non-polyposis colorectal cancer, (*b*) to establish selection criteria for patients in whom testing for germline mutation of the *MSH2* repair gene should be performed.
- 164 patients treated for sporadic upper urinary tract transitional cell carcinoma were screened for microsatellite instability. Twenty seven patients had high microsatellite instability levels. For those patients, we collated clinical data, and performed immunohistochemistry to investigate loss of hMSH2 protein and PCR single strand conformation polymorphism gene sequence analysis to detect *hMSH2* mutations.
- The presence of a mutation was significantly related to (*a*) a history of a HNPCC associated cancer ($p=0.038$), (*b*) the occurrence of upper urinary tract transitional cell carcinoma before 60 years of age ($p=0.04$), and (*c*) the indication by loss of protein expression on immunohistochemistry.
- In cases of upper urinary tract transitional cell carcinoma with high microsatellite instability levels, hereditary predisposition should be investigated if the patient has a history of a HNPCC associated cancer or is under 60. Patients with hMSH2 protein loss on immunohistochemistry should undergo testing for a germline mutation.

Abbreviations: HNPCC, hereditary non-polyposis colorectal cancer; PCR, polymerase chain reaction; SSCP, single strand conformation polymorphism; UUTTCC, upper urinary tract transitional cell carcinoma

study has already been performed in high risk colorectal cancer patients.[18]

PATIENTS AND METHODS

Patients

Our three urology departments treated a total of 164 patients for sporadic upper urinary tract transitional cell carcinoma between 1990 and 2002. None of these patients met the Amsterdam clinical criteria for hereditary non-polyposis colorectal cancer (Criteria I before 1999, Criteria II after 1999)[5] (table 1). The formalin fixed, paraffin embedded primary tumour blocks of these patients were retrieved from our archives and a sample was used to screen for microsatellite instability. All patients with high microsatellite instability levels were selected and the following data were collated: patient age, personal or family history of a possibly HNPCC-associated tumour, history of other cancers, tumour stage (1997 TNM[19] and WHO[20] classifications) and grade.

DNA extraction

Paired normal blood and tumour DNA was extracted. Tumour DNA was extracted from 40 µm tumour sections using Qiagen tissue and blood kits (Qiagen, Valencia, CA) according to the manufacturer's instructions. DNA concentration was measured spectrophotometrically (DU 640 B, Beckman Instruments, CA) at 260 nm and adjusted to 200 ng/µl.

Screening for microsatellite instability

Paired DNA from tumour and normal tissue was amplified by PCR using 16 microsatellite markers: BAT25 (4q12), BAT26 (2p16), MFD15 (1q23), D2S123 (2p16), APC (5q22), BAT40 (1p13.1), D18S58 (18q22), D18S69 (18q21), D10S197 (10p12), MYC1L (1p34), UT5320 (8q24), ACTBP2 (6q13), CFS1R (5q33-q35), D20S82 (20p12), D11S488 (11q24), and D9S242 (9q33). PCR amplification was performed with approximately 10 ng of DNA in a 20 µl final volume of reaction mixture (0.25 mM dNTP in 1 M Tris; 0.9 M boric acid; 0.01 M EDTA; 20 pmol of each primer (MWG Biotech, Ebersberg, Germany); 0.75 µl DMSO; 1 U Taq Polymerase (Qbiogen, Illkirch, France)). Cycling parameters are described elsewhere.[21] One microlitre of PCR product was added to 1 µl of blue Dextran and 3 µl of formamide. Immediately after a 2 min denaturation step at 94℃, the mixture was immersed in an ice bath. The amplified fragments were separated by denaturing gel electrophoresis (TBE buffer/4% polyacrylamide (Acryl/Bisacryl 29/1) 6 M urea gel) using an ABI PRISM 377 Genetic Analyzer® (Applied Biosystems, Palo Alto, CA). For data analysis, we used GeneScan 3.1 Fragment Analysis Software® (licensed by Applied Biosystems, Palo Alto, CA).

The microsatellite instability level was defined as high (4/15 markers unstable) in accordance with the criteria of the National Cancer Institute consensus on microsatellite instability.[22]

Table 1 Amsterdam criteria I and II

Amsterdam criteria I:
1 One member diagnosed with colorectal cancer before age 50.
2 Two affected generations.
3 Three affected relatives, one of them a first degree relative of the other two.
4 Familial adenomatous polyposis should be excluded.
5 Tumours should be verified by pathological examination.
Amsterdam criteria II:
1 There should be at least three relatives with an HNPCC-associated cancer (colorectal cancer, cancer of the endometrium, small bowel, ureter, or renal pelvis).
2 One should be a first degree relative of the other two.
3 At least two successive generations should be affected.
4 At least one should be diagnosed before age 50.
5 Familial adenomatous polyposis should be excluded in the colorectal cancer cases.
6 Tumours should be verified by pathological examination.

Immunohistochemistry

Staining for hMSH2 was performed on 5 µm tissue sections from the tissue array block, placed on charged slides. Slides were deparaffinised, rehydrated, and pretreated by microwave for antigen retrieval. The primary antibody was a mouse monoclonal antibody directed against hMSH2 (Oncogene Research product, Cambridge, MA), used at 1/200 dilution and incubated for 30 min. Immunostaining was performed using the Ventana ES automated immunohistochemistry system (Ventana Medical Systems, Strasbourg, France). The automated procedure is based on an indirect biotin-avidin system with a universal biotinylated immunoglobulin secondary antibody, diaminobenzidine substrate, and haematoxylin counterstain. A negative control slide was incubated with an irrelevant antibody (mouse monoclonal immunoglobulin, Ventana Medical Systems).

PCR, single strand conformation polymorphism, and sequencing

The *hMSH2* gene was analysed for mutations using a combination of polymerase chain reaction (PCR) and single strand conformation polymorphism (SSCP), as described previously.[23] A sequence analysis of genomic DNA was performed only on exons with aberrant SSCP banding. DNA was isolated from peripheral blood lymphocytes using a purification kit (QIAamp blood kit, Qiagen, Courtaboeuf, France). All coding exons of the *hMSH2* genes were sequenced by PCR amplification with intronic flanking primers. Primers were designed using the human genome sequence (Genbank). Sequencing reactions were performed with the ABI Prism Big Dye Terminator Cycle sequencing kit and analysed on an ABI310 sequence analyser (both from Applied Biosystems, Foster City, CA).

Statistical analysis

We used Statview© (Abacus Concepts, Inc., Berkeley, CA, 1996) for statistical tests. The χ^2 test was used to compare the prevalence of *hMSH2* mutations in patients with and without a history of cancer. A logistic regression test was used to compare patients with upper urinary tract transitional cell carcinoma before and after 60 years of age. A p value <0.05 was considered significant.

RESULTS

Phenotype

Of the 164 patients, 27 (19 men, 8 women) had tumours with a high microsatellite instability level (16%). Their sex, age, personal and family history, and tumour characteristics are given in table 2. The mean age at upper urinary tract transitional cell carcinoma diagnosis was 70.1 (8) years (range 54–90). Four patients were under 60 years old (mean 55.8 (2.1) years; range (54–58). Seven patients had a personal history of cancer; one of them had presented with two separate primary cancers (first colon cancer, then breast cancer). Six of these eight cancers (5 colon, 1 endometrium) belonged to the HNPCC spectrum of cancers. Upper urinary tract transitional cell carcinoma was never the first cancer in their personal history. No patient had a family history of HNPCC-associated cancer.

Of the 27 patients, 11 developed upper urinary tract transitional cell carcinoma in the renal pelvis and 16 in the

ureter. No patient presented metastases at the time of diagnosis of upper urinary tract transitional cell carcinoma. Of the 27 tumours, 11 were superficial (pTa, pT1) and 16 were invasive (pT2, pT3); 21 were N0, 5 were N1, and 1 was N2; 1 was grade 1, 15 were grade 2, and 11 were grade 3. The upper urinary tract transitional cell carcinoma was multifocal and synchronous with bladder carcinoma in three patients. All 27 patients underwent radical nephro-ureterectomy; 8 experienced recurrence (5 bladder cancers, 3 cancers of the contralateral upper urinary tract).

Genotype and immunohistochemistry

There was a mutation of the *MSH2* gene in 3 (11%) of the 27 patients with a high microsatellite instability status. In the immunohistochemistry test, the tumours of 10 patients showed normal hMSH2 protein expression. On the other hand, 15 of the 25 patients for whom results were obtained showed loss of hMSH2 protein expression (table 2). A gene mutation was found in 3 of these 15 patients.

There was a significant relationship between the presence of a mutation and (*a*) history of a HNPCC associated cancer ($p = 0.038$), (*b*) occurrence of upper urinary tract transitional cell carcinoma before 60 years of age ($p = 0.04$). For these three patients, there was also a significant relationship between the *MSH2* mutation and loss of protein expression ($p<0.0001$) on immunohistochemistry.

However, for other patients, there was no significant relationship with tumour stage, grade, multifocal nature or loss of protein expression on immunohistochemistry.

DISCUSSION

Application of the stringent clinical criteria for the diagnosis of hereditary non-polyposis colorectal cancer (Amsterdam criteria[3]) reveals that 6% of colorectal cancers are hereditary. Microsatellite instability screening identifies a further 3% of hereditary cancers.[2] In addition, the incidence of de novo *hMSH2* mutations is not negligible.[24 25] Consequently, some hereditary cancers, whether colon cancer or upper urinary tract transitional cell carcinoma, are misclassified as sporadic and their incidence is underestimated.[2 26]

The tumours of 27 (16%) of our 164 patients with sporadic upper urinary tract transitional cell carcinoma, had high microsatellite instability levels. Three of the 27 patients had a previously undetected mutation of the *hMSH2* gene, which is the mismatch repair gene most often affected in hereditary non-polyposis colorectal cancer. There was a significant relationship between the presence of the mutation and (*a*) history of a HNPCC-associated cancer ($p = 0.038$) and (*b*) upper urinary tract transitional cell carcinoma occurrence before 60 years of age ($p = 0.04$). Screening for microsatellite instability is thus a useful means of detecting hereditary cancers, and a routine screen for microsatellite instability seems warranted in all patients with upper urinary tract transitional cell carcinoma, as for colorectal cancers, irrespective of age at diagnosis. For effective screening, we used a panel of 15 microsatellite markers rather than the usual procedure of using first 5 markers, then a further 10 if the first 5 did not yield discriminating results.[22 26]

Upper urinary tract transitional cell carcinoma was never the first cancer in the history in our 27 patients. Yet, in one out of every two patients, upper urinary tract transitional cell carcinoma reveals the presence of hereditary non-polyposis colorectal cancer and, conversely, the relative risk of upper urinary tract transitional cell carcinoma in hereditary non-polyposis colorectal cancer patients in the literature is 14.[4 6 27]

The mean age of upper urinary tract transitional cell carcinoma diagnosis is between 65 and 70 years of age.[1 4 27] Our three patients with a mutation of the *hMSH2* gene were younger (mean 55.8 years). In order not to overlook a hereditary cancer, we suggest that all patients with a high microsatellite instability level and who meet one of the Amsterdam criteria or are under 60 years of age should be tested for germline mutations (fig 1). In the case of absence or low levels of microsatellite instability, this test is unwarranted.

Table 2 Characteristics of patients with upper urinary tract transitional cell carcinoma displaying high microsatellite instability levels

			History of cancer				Stage		hMSH2	
No	Sex	Age, years	Personal	Family	UUTTCC site	Grade	pT	TNM	Protein loss	Gene mutation
1	F	58	Colon, breast	No	Renal pelvis	2	1	T1N0M0	+	811 del 4
2	M	71	Colon	No	Ureter	3	2	T2N0M0	–	–
3	F	74	No	No	Ureter	2	a	TaN0M0	+	–
4	M	70	No	No	Renal pelvis	2	2	T2N0M0	+	–
5	M	57	Colon	No	Renal pelvis	2	2	T2N0M0	+	R711X
6	F	70	No	No	Renal pelvis	3	2	T2N0M0	+	–
7	M	71	No	No	Ureter	2	1	T1N0M0	–	–
8	M	74	No	No	Renal pelvis	2	2	T2N0M0	+	–
9	M	73	No	No	Ureter	3	2	T2N0M0	+	–
10	M	65	No	No	Renal pelvis	3	2	T2N0M0	+	–
11	F	73	No	No	Ureter	3	2	T2N1M0	+	–
12	M	72	No	No	Ureter	3	2	T2N0M0	NA	–
13	M	66	No	No	Ureter	2	2	T2N0M0	NA	–
14	M	73	Colon	No	Renal pelvis	3	3	T3N1M0	+	–
15	M	70	Prostate	No	Renal pelvis	3	3	T3N2M0	+	–
16	M	69	Colon	Mother (breast)	Ureter	2	1	T1N0M0	–	–
17	M	71	No	No	Renal pelvis	2	3	T3N1M0	+	–
18	M	81	No	No	Ureter	3	3	T3N1M0	–	–
19	M	74	No	No	Renal pelvis	2	3	T3N1M0	–	–
20	M	90	No	No	Ureter	3	2	T2N0M0	–	–
21	F	68	No	No	Ureter	2	a	TaN0M0	–	–
22	F	80	No	No	Ureter	3	a	TaN0M0	–	–
23	M	73	No	No	Ureter	1	a	TaN0M0	+	–
24	M	77	No	No	Ureter	2	a	TaN0M0	+	–
25	M	54	No	No	Ureter	2	1	T1N0M0	–	–
26	F	66	No	No	Renal pelvis	2	a	TaN0M0	–	–
27	F	54	Endometrium	No	Ureter	2	a	TaN0M0	+	R389X

NA, not available

Immunohistochemistry is a simple method but does not always detect protein loss.[28 29] Results depend on the type of mutation or on DNA promoter hypermethylation. Moreover, some cases of protein loss could be related to a gene germline mutation because of large rearrangements or deletions that we could have missed in our study. Loss of protein expression on immunohistochemistry was associated ($p<0.0001$) with the presence of the mutation in our three patients. Consequently, we suggest that when immunohistochemistry reveals loss of hMSH2 protein expression in patients with high microsatellite instability status and early onset diagnosis of upper urinary tract transitional cell carcinoma or history of tumours related to the HNPCC spectrum, the result is followed up by DNA sequencing to detect germline mutation.

DNA sequencing is complex and expensive, and needs to be restricted to few patients[30] even though testing for mutations of the *hMSH2* and *hMLH1* genes is now available in many centres. In our three patients, it provided a firm diagnosis of hereditary cancer. Mutations in the *hMSH2* gene account for 60% of hereditary non-polyposis colorectal cancers.[2] Mutations in the *hMLH1* gene, for which we did not test, account for 30%.[1] Detection of mutations in other genes (*hMSH3*, *hPSM2*, or *hMSH6*) is not readily available. We propose the decision flow chart shown in fig 1 for the management of patients with sporadic upper urinary tract transitional cell carcinoma.

When gene mutations are detected, the patient benefits from multidisciplinary management.[4 25] The presence of other HNPCC associated cancers is sought and close monitoring of patients is undertaken. Genetic counselling is provided to the patient's family.[31]

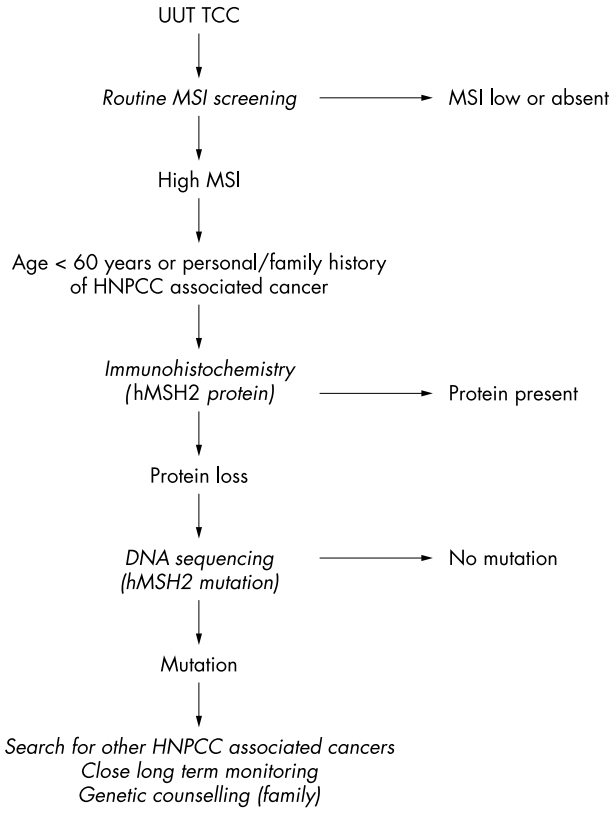

Figure 1 Flow chart for the management of sporadic upper urinary tract transitional cell carcinoma (UUT TCC).

CONCLUSIONS

Microsatellite instability testing should be sought in all patients diagnosed with upper urinary tract transitional cell carcinoma. It is positive in 40% of cases. If the microsatellite instability level is high, as in 16% of our cases, hereditary cancer may be suspected, especially if the patient is under 60 years of age or has a personal or family history of a HNPCC related cancer. When loss of hMSH2 protein expression is found on immunohistochemistry, the gene should be sequenced to detect any mutations.

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