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Modified UroVysion scoring criteria increase the urothelial carcinoma detection rate in case of equivocal urinary cytology

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Modified UroVysion scoring criteria increase the urothelial carcinoma detection rate in case of equivocal urinary cytology

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1 TITLE

2 Modified UroVysion scoring criteria increase the urothelial carcinoma detection rate in case
3 of equivocal urinary cytology

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23 **RUNNING TITLE**

24 UroVysion and equivocal cytology

25

26 **KEYWORDS**

27 Cystoscopy; cytology; FISH; urothelial carcinoma; UroVysion

28 **ABSTRACT**

29 Aim

30 UroVysion[®] is a four-target fluorescence in situ hybridisation (FISH) technique to detect
31 urothelial carcinoma (UC) in urinary cytology. The aim of this retrospective study is to
32 investigate the UC detection rate of a modified UroVysion test in patients with equivocal
33 urinary cytology. The modification comprised the addition of a cytological pre-screening
34 technique and different evaluation criteria.

35 Methods & results

36 A thin layer slide was prepared of the residual urine sample of 82 patients with equivocal
37 urinary cytology, pre-stained and pre-screened to confirm the presence of atypical urothelial
38 cells. The same slide was used for the UroVysion test and scored according to different
39 evaluation criteria. Results were compared with the outcome of cystoscopic and histologic
40 findings.

41 UroVysion detected 68% of the UCs when the manufacturer's evaluation criteria were
42 applied. In case of altered evaluation criteria, the sensitivity increased to 81% when at least
43 one copy number change of a probe target was considered as a positive test. The specificity
44 only decreased from 84% to 82%.

45 Conclusions

46 Our data suggest that the sensitivity of the UroVysion test can be increased with the addition
47 of a cytological pre-screening technique prior to the UroVysion test and a modification of the
48 UroVysion evaluation criteria.

49 **TEXT**

50 **INTRODUCTION**

51 Bladder cancer is the seventh most common cancer worldwide. It accounts for about 3.2% of
52 all cancers, mostly urothelial carcinomas (UC). The highest prevalence of bladder cancer is
53 observed in countries of the European Union and North America [1].

54 Bladder cancer is mostly detected by cystoscopy combined with urinary cytology. Cystoscopy
55 is very successful in detecting low-grade and high-grade papillary tumours but may fail to
56 identify carcinoma in situ (CIS) [2]. Cytology is a sensitive and specific examination for both
57 high-grade papillary and flat lesions, but is less sensitive for detecting low-grade papillary
58 lesions. Both techniques are thus complementary. Because cystoscopy is an invasive
59 procedure and urinary cytology has a low sensitivity for low-grade tumours, additional tests
60 emerged for the diagnosis of urothelial carcinoma (UC) on urine samples [3].

61 UroVysion[®] (UV (Abbott Molecular, Des Plaines, IL, USA)) is one of the most common used
62 commercial UC markers [3]. It is a multi-target, multi-colour fluorescence in situ
63 hybridisation (FISH) assay that detects aneusomy of chromosomes 3, 7 and 17, and loss of
64 9p21. UV has an overall sensitivity of 72% and specificity of 83% [4], moreover, it remains
65 sensitive in the follow-up of patients under Bacillus Calmette-Guérin (BCG) treatment [3, 5-
66 7]. Recent studies suggested the use of more flexible evaluation criteria for the UV test
67 leading to an increase of the sensitivity of the UV test.[8-9].

68 When the UV test was introduced at our department, we added a pre-screening technique to
69 the test. A FISH test is normally performed on another slide than the original slide used for
70 cytological screening. This second slide may not always contain the atypical cells present in
71 the previous cytological slide. In order to prevent false negative results and to confirm the
72 presence of atypical cells also in the second slide, the UV test was preceded by a pre-staining
73 and cytological screening of this slide (figure 1).

74 In the current study we retrospectively investigated the sensitivity of the UV test in
75 combination with the pre-screening method for the detection of UCs in patients with
76 equivocal urinary cytology. Additionally, the impact of different evaluation criteria [8-9] on
77 the sensitivity of the UV test was examined.

78

79 MATERIAL AND METHODS

80 **Patient population**

81 In this study we retrospectively included patients with equivocal urinary cytology for which
82 UV tests and follow up examinations were performed. Data were collected from December
83 2005 till May 2009 (Table 1). Equivocal cytology was defined as cytology with a diagnosis
84 that was neither clearly negative nor clearly positive for malignancy. The study group
85 contained patients in the follow-up for UC, who all received intravesical chemotherapy or
86 BCG, as well as patients without a history of UC. Cystoscopy and histology were used as
87 'gold standards' to evaluate the presence of UC. The histology was evaluated and graded in
88 accordance with the 1999 World Health Organization classification. The tumour stage was
89 assigned according to the TNM classification, 6th edition. Cystoscopy was regarded as
90 positive in case of an unequivocal tumour mass. In this study patients without information
91 about a consecutive cystoscopy or biopsy, as well as patients with FISH specimens lacking
92 the atypical cells were not included.

93

94 **Urinary cytology**

95 The urine samples were fresh voided (n=63), obtained through bladder washings (n=8),
96 bladder (n=7) or ureter (n=4) catheterizations and collected in 50% ethanol. They were poured
97 in a 50 ml conical centrifuge tube and centrifuged at 1000 rpm during 10 min. The cell pellet
98 was added to 5 ml Cytoschick[®] Blue (BD Diagnostics TriPath, Burlington, NC, USA). Slides for

99 cytologic evaluation were made using the thin layer technique (BD Diagnostics Prepstain[®]
100 slide processor, Burlington, NC, USA) and stained with the Papanicolaou stain.

101

102 **UroVysion FISH**

103 *Pre-screening technique*

104 A second thin layer preparation was stained with haematoxylin for cytological pre-screening.

105 Atypical cells were scored using a Leica DM5000B microscope in bright field mode and their

106 location annotated by coordinates. Cases with slides that contained no or a too low number of

107 atypical cells **to successfully score the UV evaluation criteria (see further)** were not included

108 in the study. The pre-screening technique was tested prior to the study and resulted in good

109 quality of hybridization on the pre-stained specimens.

110 *FISH*

111 After cytological pre-screening, the slides were heated at 65°C for 30 min and immersed in a

112 pepsin solution (500 mg/l, 10mM HCl), for 7 min. After washing in phosphate-buffered saline

113 (PBS) cells were postfixed in 1% formaldehyde with 20mM MgCl₂ in PBS for 5 min,

114 dehydrated in 70%, 96% and 2 X 100% ethanol and air dried. Six µl UroVysion probe

115 mixture, containing probes for the chromosome 3, 7 and 17 centromeric regions and the 9p21

116 locus labelled with SpectrumRed, SpectrumGreen, SpectrumAqua and SpectrumGold

117 respectively, was administered under a coverslip of 18 x 18 mm. Cellular and probe DNA

118 were denatured at 73°C for 2 min in a ThermoBrite[®] system (Abbott Molecular, Des Plaines,

119 IL, USA). Posthybridisation washings were made in 0.4x SSC/0.3% 4-

120 nonylphenolpolyethyleneglycol (NP-40, Abbott Molecular, Des Plaines, IL, USA) for 2 min

121 at 73°C. The slides were rinsed in 2x SSC/0.1% NP-40 at room temperature for 2 min, air

122 dried , and counterstained with 4,6-diamidine,2-phenylindole dihydrochloride (DAPI)

123 solution. Next, the slides were examined using the Leica DM5000B microscope with

124 appropriate fluorochrome filter sets and evaluation included the atypical cells at the annotated
125 coordinates. The UV test was considered positive if 1) four or more cells out of a minimum of
126 25 morphologically abnormal cells demonstrated polysomy in two or more chromosomes (3,
127 7 and 17) in the same cell and/or if at least 12 morphologically abnormal cells demonstrated
128 homozygous loss of 9p21 (= UV1 criteria, according to Abbott Molecular); or 2) at least one
129 chromosomal gain (3, 7 or 17) was detected in four or more cells out of a minimum of 25
130 morphologically abnormal cells and/or if at least 12 morphologically abnormal cells
131 demonstrated heterozygous or homozygous loss of 9p21 (= UV2 criteria).

132

133 **Statistics**

134 The chi-square test was used to evaluate the relation between the test results (UV and
135 cystoscopy) and the presence of UC. All tests were 2-tailed with $p < 0.05$ considered
136 statistically significant. Statistical analysis was performed with SPSS® for Windows, version
137 15.0.

138

139 **RESULTS**

140 Table 1 shows the patient characteristics including the number of the cystoscopic
141 examinations, performed simultaneously with or within three months after the urine
142 collection, and histologic evaluations, executed within an average of four months. Thirty-one
143 out of the 82 patients revealed a UC on histology and / or cystoscopy. In seven out of 31
144 patients with UC no biopsy was taken because of clearly presence of tumour on cystoscopy.
145 In six of these patients, there was already a prior history of UC.

146 The application of the UV1 criteria resulted in an overall sensitivity of 68% . Using the UV2
147 criteria the sensitivity increased to 81%, while the specificity only decreased from 84% to
148 82%. Very high sensitivity scores were realised when combining UV and cystoscopy (Table

149 2).

150 Sensitivity and specificity of the UV test in follow-up patients, who all received intravesical
151 chemotherapy or BCG, were 75% and 84%, respectively, using the UV1 criteria, in contrast
152 with 88% and 81%, applying the UV2 criteria. For patients without a history of UC, UV1
153 criteria resulted in a sensitivity and specificity of 60% and 84%, respectively, compared to
154 73% and 84% in case of applying UV2 criteria.

155 Cystoscopic evaluation alone had a sensitivity of 65%: 11 out of 62 patients with negative
156 cystoscopies revealed a UC on biopsy. In 7/11 cases the UC was located in the upper urinary
157 tract (UUT). The other four cases were three CIS lesions and one grade 3 UC of the bladder.
158 Out of 20 UCs detected on cystoscopy, the UV test was negative in six or seven cases,
159 depending on using the UV2 or UV1 criteria.

160 When applying the UV1 criteria, UV detected 0/3 grade 1 UCs and only 1/5 grade 2 UCs,
161 while cystoscopy was positive in 3/3 grade 1 UCs and 3/5 grade 2 UCs (Table 3). For grade 3
162 lesions and carcinoma in situ (CIS) UV was positive in respectively 10/12 and 4/4 lesions,
163 while cystoscopy detected six grade 3 lesions and only one CIS. It is important to mention
164 that of the cystoscopically missed malignancies, all two grade 2 lesions and 5/6 grade 3
165 lesions were located in the UUT. Regarding the relationship between tumour stage, UV and
166 cystoscopy, there are no significant differences between the detection rates of UV and
167 cystoscopy (Table 3). Exceptions are T3 tumours where none of the lesions were detected by
168 cystoscopy, however these tumours were UUT tumours.

169 Of the 31 patients with a proven UC, chromosomal alterations were found in 25 cases. Gain
170 of chromosome 3 (18/31), 7 (19/31) and/or 17 (15/31) was noted more frequently than
171 heterozygous (4/31) or homozygous (9/31) deletions of locus 9p21. Interestingly, all CIS
172 lesions had a gain of chromosome 3, 7 and 17. Five patients had chromosomal alterations that
173 did not fit the UV1 criteria: one patient with an isolated gain of chromosome 7, three patients

174 with an isolated heterozygous deletion of 9p21 and one patient with an isolated heterozygous
175 deletion of 9p21 combined with a solitary gain of chromosome 3.
176 In the patients with UUT tumours, the UV test was positive in 5/7 cases with the UV1 criteria
177 and in 7/7 cases with the UV2 criteria. Chromosomal alterations in the UUT tumours were not
178 significantly different from the bladder UCs.

179

180 DISCUSSION

181 Currently, cystoscopy and urinary cytology are routinely used for the detection of tumours in
182 the urinary tract. However, on cytology, it is sometimes difficult to discriminate between
183 reactive and malignant atypical urothelial cells. Urothelial cells can show reactive atypia due
184 to instrumentation, inflammation, infection, calculi, radiotherapy and intravesical BCG or
185 chemotherapy [10-14]. Atypical findings are problematic because they raise concerns with the
186 patient and physician about the possible presence or recurrence of cancer [15]. The UV test,
187 however, can be a helpful additional tool in those cases [16, 17].

188 The present study evaluates the combination of the UV test with a cytological pre-screening
189 on the same slide. This method allows us to link cytological observed cellular atypia with
190 chromosomal alterations. It can be difficult to select the atypical cells in a FISH specimen in
191 the case of equivocal urine cytology. The pre-screening technique however can facilitate this.

192 When gain of at least one chromosome (3, 7 or 17) and/or heterozygous or homozygous
193 deletion of 9p21 (UV2 criteria) were interpreted as a positive UV test, 81% of the
194 malignancies were detected compared to 68% when applying the manufacturer's criteria
195 (UV1 criteria) with only a slight decrease in specificity (2%). Our data indicate that in case of
196 equivocal urinary cytology the use of more flexible criteria for UV positivity substantially
197 increases the number of detected UCs. This suggests that the current scoring criteria may be
198 too restrictive since some urothelial carcinomas have chromosomal abnormalities (gains of

199 single chromosomes or heterozygous 9p21 deletions) that are undetected by the
200 manufacturer's criteria.

201 Until now, no study has compared the current manufacturer's UV criteria with more flexible
202 criteria in urine specimens of patients with equivocal cytology by using a cytological pre-
203 screening technique in combination with the UV test. Ferra et al [9] also compared more
204 flexible evaluation criteria, i.e. any cytogenetic abnormality regardless of the number of cells
205 is a positive result, with the manufacturer's criteria in equivocal urinary cytology cases. With
206 a sensitivity of 68% and 83% by using respectively the manufacturer's criteria and the
207 flexible criteria, their results are almost identical to ours. Nevertheless, their specificity
208 dropped from 40% to 22%, while we maintained a specificity of more than 80%. This
209 difference might be explained by the pre-screening technique we used, as it allows us to better
210 allocate the exact location of the atypical cells before application of the UV test, thereby
211 excluding that tetrasomic cells, such as umbrella cells or dividing cells, are scored positive [8,
212 18]. Also the number of the present atypical cells and cells with cytogenetic abnormalities
213 needs to be high enough to maintain a high specificity.

214 Furthermore, in a recently published analysis of 14 studies involving 2477 UV tests (using
215 UV1 criteria) the pooled sensitivity and specificity was 72% and 83% [4]. The overall
216 prevalence of UCs in the examined patient groups was 35%, almost identical to the 36% in
217 our study. In these studies the UV test was not only performed on equivocal urine specimens,
218 but also on cytologic clear negative or positive cases. The fact that the sensitivity and
219 specificity scores of these studies are comparable with our UV1 data, indicates that the pre-
220 screening technique we use to trace atypical cells, leads to a more effective application of the
221 UV test.

222 The UV test in our series detected 7 UUT tumours, most of them in spontaneously voided
223 urine specimens. This finding suggests that the UV test can be helpful in cases of equivocal

224 cytology and negative findings on cystoscopy, which is in agreement with other studies [19-
225 21].

226 Furthermore, our results show that the UV test and cystoscopy are complementary techniques,
227 achieving extremely high sensitivity rates when both are executed (Table 2).

228 In accordance with other reports [3, 5-7] this study also confirms that the UV test can be
229 performed on urine specimens of follow-up patients, who received intravesical therapy.

230 Sensitivity scores in our study are even higher in this population than in those without a
231 tumour history.

232 There are some hypotheses for the false negative UV cases we encountered. Firstly, UCs in
233 these cases may have other chromosomal alterations than those detectable by UV. Secondly,
234 it is also known that low grade UCs are mostly diploid tumours [22, 23] and this may explain
235 the false negative results, since most of our false negative UV cases were low grade tumours.

236 What to do with a positive UV test without detectable UC? In this regard, a positive UV test
237 has been shown to be predictive of a future recurrence [8, 24-25] as it may be an indicator of
238 unstable urothelium capable of or primed for malignant transformation, thus detecting patients
239 at significant risk [26]. However, tetrasomic cells, mostly umbrella cells or dividing cells, can
240 be present in urine samples and should not be interpreted as FISH-positive, malignant cells [8,
241 18].

242 In summary, our results suggest that a modification of the standard UV evaluation criteria on
243 pre-screened preparations can result in a higher detection rate of UCs in patients with
244 equivocal urinary cytology. Future prospective, blinded studies may help to validate our
245 results in a higher number of patients and on a diversity of specimens (voided urine
246 specimens, bladder washings, bladder and ureter catheterizations).

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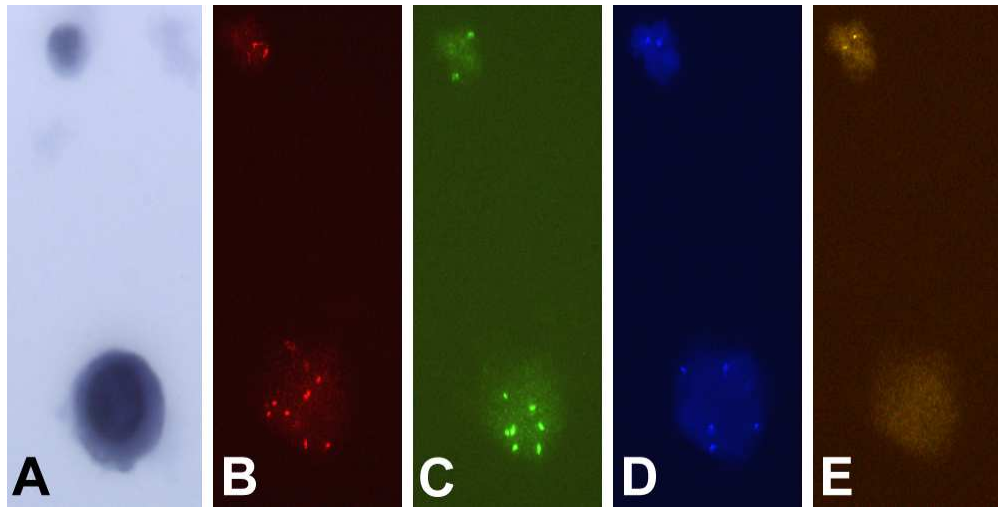
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Figure 1. The coordinates of the atypical urothelial cell (A, under) on a haematoxylin pre-stained preparation are annotated during prescreening. Note the neutrophilic granulocyte (A, above). After fluorescence in situ hybridization the same atypical urothelial cell shows 7 copies of the centromeric region of chromosome 3 labeled with the fluorophore SpectrumRed (B), 7 copies of the centromeric region of chromosome 7 labeled with SpectrumGreen (C), 5 copies of the centromeric region of chromosome 17 labeled with SpectrumAqua (D) and homozygous loss of 9p21 labeled with SpectrumGold (E), while the neutrophilic granulocyte keeps 2 copies of each locus.

1 Table 1: Patient characteristics

Number of patients	82
<ul style="list-style-type: none"> • No history of UC • History of UC <ul style="list-style-type: none"> ○ Bladder UC ○ Ureter UC ○ Bladder + ureter UC 	<ul style="list-style-type: none"> • 34 • 48 <ul style="list-style-type: none"> ○ 45 ○ 1 ○ 2
Patients with cystoscopic evaluation	82
Patients with histologic evaluation	36
Male/female	4.1/1
Median \pm SD patient age (years)	70 \pm 11.4

2 UC: urothelial carcinoma

Table 2: Cystoscopy and UV test performance characteristics for UC detection in patients with equivocal cytology specimens (n = 82 patients)

Examination	Sens	Spec	PPV	NPV	p
Cystoscopy	65% (20/31)	NA	NA	82% (51/62)	p<0.0001
UV1	68% (21/31)	84% (43/51)	72% (21/29)	81% (43/53)	p<0.0001
UV2	81% (25/31)	82% (42/51)	74% (25/34)	88% (42/48)	p<0.0001
UV1/cysto+	90% (28/31)	84% (43/51)	78% (28/36)	94% (43/46)	p<0.0001
UV2/cysto+	100% (31/31)	82% (42/51)	78% (31/40)	100% (42/42)	p<0.0001

UV: UroVysion® / UC: urothelial carcinoma

Sens: sensitivity / Spec: specificity

PPV: positive predictive value / NPV: negative predictive value

UV1: UV test positive according to the strict manufacturer's criteria

UV2: UV test positive when polysomy in at least one chromosome (3, 7 or 17) and/or heterozygous or homozygous deletion of 9p21

UV1/cysto+: UV test positive according to the strict manufacturer's criteria and/or cystoscopy positive

UV2/cysto+: UV test positive when polysomy in at least one chromosome (3, 7 or 17) and/or heterozygous or homozygous deletion of 9p21 and/or cystoscopy positive

NA: not applicable, since cystoscopy was used as gold standard in case of no histology control

1 Table 3: Relationship between tumour grade, stage, cystoscopy and UV test

Patients with equivocal cytology and histology-proven UC (n = 24 patients)								
	G1 (n=3)	G2 (n=5)	G3 (n=12)	CIS (n=4)	Ta (n=15)	T1 (n=3)	T2 (n=1)	T3 (n=5)
Cysto-	0	2 ^a	6 ^b	3	5 ^c	1 ^d	0	5 ^e
Cysto+	3	3	6	1	10	2	1	0
UV-	3	1	1	0	5	0	0	0
UV1	0	1	10 ^b	4	8 ^c	3 ^d	1	3 ^f
UV2	0	4 ^a	11 ^b	4	10 ^c	3 ^d	1	5 ^e
UV1/cysto+	3	3	11 ^b	4	14 ^c	3 ^d	1	3 ^f
UV2/cysto+	3	5 ^a	12 ^b	4	15 ^c	3 ^d	1	5 ^e

2 **UC**: urothelial cell carcinoma / CIS: carcinoma in situ

3 ^a: incl 2 pyelum **UCs** / ^b: incl 2 ureter **UCs** and 3 pyelum **UCs** / ^c: incl 1 pyelum **UC** / ^d: incl 1

4 ureter **UC** / ^e: incl 1 ureter **UC** and 4 pyelum **UCs** / ^f: incl 1 ureter **UC** and 2 pyelum **UCs**

5 Cysto-: cystoscopy negative / Cysto+: cystoscopy positive

6 UV-: UV test without chromosomal alterations

7 UV1: UV test positive according to the strict manufacturer's criteria

8 UV2: UV test positive when polysomy in at least one chromosome (3, 7 or 17) and/or

9 heterozygous or homozygous deletion of 9p21

10 UV1/cysto+: UV test positive according to the strict manufacturer's criteria and/or cystoscopy
11 positive

12 UV2/cysto+: UV test positive when polysomy in at least one chromosome (3, 7 or 17) and/or
13 heterozygous or homozygous deletion of 9p21 and/or cystoscopy positive

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 Contributor address: Kwademeerslaan 19 8531 Hulste BELGIUM
 Manuscript number (if known): HISTOP-08-10-0444
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