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Research Paper

Immunohistochemical and molecular characterizations in urothelial carcinoma of bladder in patients less than 45 years

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Abstract

Bladder tumours in early-onset patients are rare and seem to exhibit unique clinicopathological features. Only few studies have investigated somatic alterations in this specific age of onset group and evidence is accumulating of a distinct molecular behaviour of early-onset bladder tumours. We collected the largest cohort of early-onset tumours of patients 45 years old or younger and aimed to test genomic alterations typically found in bladder cancer. Tumours of 118 early-onset patients were compared with a consecutive group of 113 cases. Immunohistochemistry of TP53, CK20 and Ki-67 was carried out. Molecular analysis was conducted to test for loss of heterozygosity of chromosome 9 and 17, as well as *TP53* and *FGFR3* mutations. Fisher´s exact and chi-squared test were appropriately used. No differences in grade/stage characteristics were observed. Overexpressed TP53 was differentially distributed between the two groups. TP53 nuclear accumulation was significantly more frequent in early-onset papillomas, PUNLMPs and pTa low-grade tumours compared to the consecutive cohort (p=0.005). Moreover, chromosome 9 deletions (29.5% vs. 44.6%) and *FGFR3* mutations (34.5% vs. 63.7%) were less often detected in early-onset patients (p=0.05 and p<0.0001). By comparing the largest cohort of early-onset bladder cancer patients with an unselected group, we demonstrated that the typical molecular features are not independent of age at diagnosis. Our study supports the hypothesis of a distinct biological behaviour in early-onset tumours.

Key words: Early-onset; Bladder cancer; *FGFR3*; TP53 positivity; Mutation analysis.

Introduction

Urothelial bladder cancer (UBC) usually occurs late in life, with a mean age at diagnosis of approximately 67 years [1]. UBC is a heterogeneous disease, both clinically and molecularly. Low-grade non-muscle invasive tumours are associated with activating mutations of *FGFR3* and *PIK3CA* and a lower risk of progression, whereas *TP53* and *RB* pathway alterations play a key role in the development of high-grade non- and muscle-invasive UBC [2-5]. Additionally, total or partial loss of chromosome 9 is found in almost half of the tumours of all stages and grades [6, 7].

Worldwide estimates of cancer incidences for 2012 indicate that the proportion of UBC in patients younger than 45 years is at about 3.4% [8]. Because of these low incidence rates and since no uniform threshold is used for young patients, the natural history is still under debate. However evidence is accumulating, that especially tumours of patients of the first two decades of life are more frequently low-grade/-stage with a favourable prognosis [9]. Besides distinct clinicopathological features, very few studies of paediatric and adolescent patients reported, that molecular alterations in paediatric and adolescent patients were much rarer compared to typical UBC samples [10-14]. These unexpected results gave evidence, that tumours of this age of onset group may represent a distinct biological entity. However, only small sample-sized studies were reported and thus these findings have still to be validated.

The aim of our study was to investigate specific molecular alterations of UBC in the largest cohort to date of early-onset patients, defined herein for the study as patients aged 45 or younger. Immunohistochemical analysis was performed to detect CK20, Ki-67 and TP53 expression levels. In addition, we investigated the frequencies of loss of heterozygosity (LOH) of chromosome 9 and 17. Moreover, molecular analysis revealed mutations in the *FGFR3* and *TP53* gene. All obtained results were compared with a consecutive, unselected cohort of patients.

Materials and methods

Tumour samples

118 patients aged 45 or younger were identified from 1991 until 2001 from the files of the contributing Institutes of Pathology. 113 consecutive cases were included from the period between 1996 and 2002. For each case, formalin-fixed paraffin-embedded primary UBC and normal tissues were available and reclassified according to the 2016 WHO classification by two experienced uropathologists (A.H., J.G.) [15]. Figure 1 demonstrates representative images of the histopathological specimens of early-onset patients. Eight urothelial papillomas and ten samples of papillary urothelial neoplasia of low malignant potential (PUNLMP) were included. Due to similar molecular features reported, they were grouped together with pTa low-grade tumours [16]. Table 1 shows the characteristics of the two cohorts. Institutional Review Board approval (University Hospital Erlangen) was obtained for molecular analysis on archival material.

Table 1. Clinicopathological characteristics of the two different cohorts.

Characteristics	Early-onset group		Consecutive group		Group comparison
	(n=118)		(n=113)		*p*-value
Age distribution, yr					
Minimum/maximum	17/45		48/87		
Mean ± SD	37.6 ± 6.5		69.8 ± 8.4		
Median age	39		70		
Age categories, yr					
≤ 20	3		-		
21-25	4		-		
26-30	8		-		
31-35	23		-		
36-40	32		-		
41-45	48		-		
>45	-		113		
Gender, No.					
Male	92	(79.3)	89	(78.8)	*p*=1.0
Female	24	(20.7)	24	(21.2)	
Not available	2		0		
Stage/grade distribution I, No.					
Urothelial papilloma	7		0		
Inverted urothelial papilloma	0		1		
PUNLMP	0		10		
pTa low-grade	56		62		
pTa high-grade	20		9		
pT1 low-grade	4		1		
pT1 high-grade	12		23		
pT2 high-grade	8		7		
pT3 high-grade	8		0		
pT4 high-grade	1		0		
Not available	2		0		
Stage/grade distribution II, No.					
Inverted/ Urothelial papilloma & PUNLMP & pTa low-grade	63	(54.3)	73	(64.6)	*p*=0.08
pTa high-grade & pT1	36	(31.0)	33	(29.2)	
≥ pT2	17	(14.7)	7	(6.2)	
Not available	2		0		

SD, standard deviation; PUNLMP, papillary urothelial neoplasm of low malignant potential.

Immunohistochemistry (IHC)

IHC of TP53, CK20 and Ki-67 were performed using whole tumour sections and an avidin-biotin peroxidase method with a 3,3'-diaminobenzidine chromatogen. According to the manufacturer's instructions, IHC was carried out in a NEXES immunostainer (Ventana, Tucson, AZ, USA).

Anti-TP53 (mouse monoclonal IgG, clone Bp53-12 (sc-263) [Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA], dilution 1:1000), anti-CK20 (mouse monoclonal IgG2a, clone IT-Ks20.8 (61026) [Progen Biotechnik GmbH, Heidelberg, Germany]; dilution 1:10) and anti-Ki-67 (mouse monoclonal IgG1, clone MIB1 (M7240) [Dako, Glostrup, Denmark], dilution 1:50) were used as primary antibodies. The slides were evaluated by one surgical pathologist (A.H.). Nuclear staining reaction for TP53 was scored from 0% to 100% in 10% increments. The intensity of the TP53 staining was recorded as negative, weak or strong. Considering a cut-off level at 10% of positive cells, three different categories were defined: ≤10% negative, >10% weak or >10% strong. Anti-Ki-67-immunostaining was scored from 0% to 5% in 1% increments and from 5% to 100% in 5% increments and cut-off level for increased proliferation was defined at 10%. Staining of CK20 as a urothelial associated marker was defined as either normal (cytoplasmic staining pattern of the superficial cells only) or aberrant (negative or more than 10% of the urothelial cells stained) according to Harnden et al. [17].

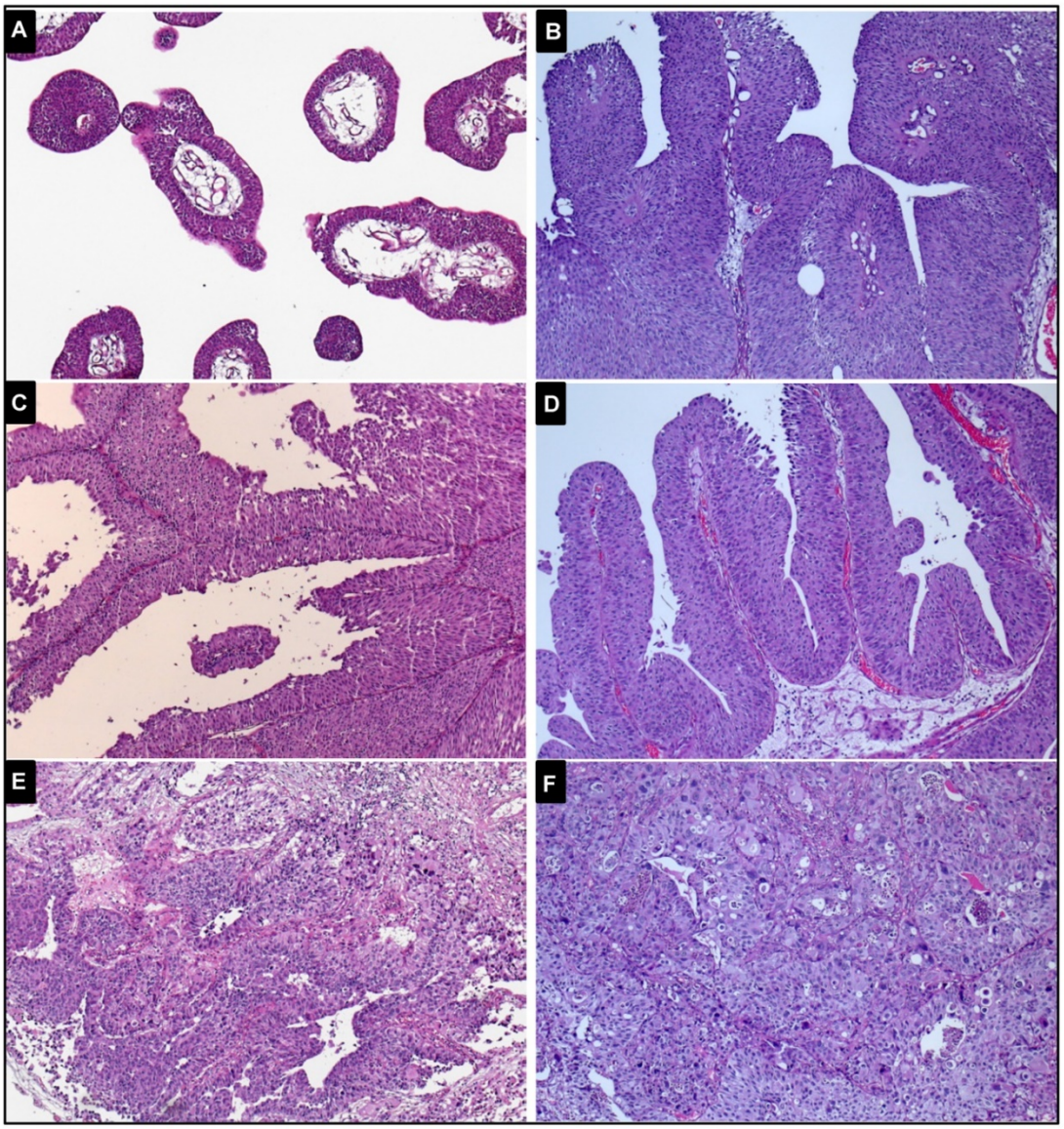

Figure 1: Representative images of histopathological specimens of the early-onset tumours (magnification: x100) A, papilloma, no analysed mutation or deletion was identified. B, pTa low-grade tumour, a *FGFR3* mutation (p.S249C) was detected. C, pTa low-grade tumour, none of the analysed alterations was observed. D, pTa high-grade tumour, a mutation of the *FGFR3* mutation (p.S249C) and LOH of chromosome 9 were observed. E, pT1 high-grade tumour, not mutated. F, pT2 high-grade tumour, a *TP53* mutation in exon 5 was identified.

Microdissection and DNA isolation

Tumour and normal DNA was isolated as described previously [18]. An experienced pathologist (A.H.) marked areas with highest tumour cell density on a representative H&E-stained section. 5-µm tissue sections were deparaffinised and tumour and non-malignant tissue were separately microdissected in sterile tubes by using sterile needles. Due to separation, tumour cells with a purity of at least 80% were obtained. DNA isolation was performed using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

Detection of LOH

For detection of LOH two microsatellite markers at chromosomal region 9p21 (D9S304, D9S1751), two markers at 9q (D9S303, D9S747) and one marker at 17p13.1 (p53Alu) were used as described previously [19]. The Polymerase chain reaction (PCR) amplification was performed in a MJ Research Thermocycler (PTC100, MJ Research, Watertown, MA, USA). By using 6.7% polyacrylamide/50% urea gel electrophoresis (1h, 1500V, 55°C) PCR products were analysed in a SequiGen sequencing gel chamber (BioRad, Hercules, CA, USA) followed by silver nitrate staining [20]. In informative cases LOH was defined as a decrease in signal intensity of the tumour sample allele to at least 50% relative to the matched normal DNA allele. All cases of LOH were confirmed at least once. Silver nitrate gels were evaluated independently by three different investigators (J.G., R.Sch., R.S.).

FGFR3 and *TP53* mutation analysis

SNaPshot method was used to analyse *FGFR3* mutations, as described previously [21]. Three regions of the *FGFR3* gene comprising the mutations were amplified simultaneously in a multiplex PCR reaction. Eight SNaPshot primers were used to detect nine mutations. An automatic sequencer (ABI Prism 310) analysed the extended primers. Direct sequencing of exons 5-9 of the *TP53* gene was assessed as described previously [22]. Identified mutations were verified in a second sequencing run to rule out polymerase errors.

Statistical analysis

Two-sided Fisher´s exact and chi-squared test were appropriately used to assess differences in the distributions of tested markers between both groups. *P*-values less than 0.05 were interpreted as statistically significant. All analyses were performed using R version 3.2.5 (www.r-project.org).

Results

Immunohistochemical analysis of CK20, Ki-67 and TP53

Table 2 summarizes the results of the IHC of CK20, Ki-67 and TP53. Neither the staining pattern of Ki-67, nor the expression of the differentiation marker CK20 showed a significant difference between the two groups. When we differentiated the aberrant staining of CK20 in negative or >10% of stained cells, we observed a significantly higher number of negative staining among the early-onset compared to the consecutive cases (p=0.03).

Table 2. Expression of CK20, Ki-67 and TP53 in the two different groups.

	Early-onset group		Consecutive group		Group comparison
IHC marker	n	(%)	n	(%)	*p*-value
Ki-67 IHC					
≤10%	63	(58.9)	55	(49.1)	p=0.19
>10%	44	(41.1)	57	(50.9)	
Not available	11		1		
CK20 IHC					
Normal	37	(37.4)	40	(35.4)	p=0.88
Aberrant	62	(62.6)	73	(64.6)	
Negative	13	(13.1)	5	(4.4)	
>10% of stained cells	49	(49.5)	68	(60.2)	
Not available	19		0		
TP53 IHC I					
≤10% negative & >10% weak	82	(73.9)	90	(79.6)	p=0.39
>10% strong	29	(26.1)	23	(20.4)	
Not available	7		0		
TP53 IHC according to grade/stage					
Inverted/Urothelial papilloma & PUNLMP & pTa low-grade					
≤10% negative & >10% weak	48	(77.4)	69	(94.5)	p=0.005
>10% strong	14	(22.6)	4	(5.5)	
Not available	1		0		
pTa high-grade & pT1					
≤10% negative & >10% weak	27	(79.4)	20	(60.6)	p=0.16
>10% strong	7	(20.6)	13	(39.4)	
Not available	2		0		
≥ pT2					
≤10% negative & >10% weak	7	(50.0)	1	(14.3)	p=0.17
>10% strong	7	(50.0)	6	(85.7)	
Not available	3		0		
TP53 IHC III					
≤10% negative	45	(40.6)	81	(71.7)	p<0.0001
>10% weak	37	(33.3)	9	(8.0)	
>10% strong	29	(26.1)	23	(20.3)	
Not available	7		0		

IHC, immunohistochemistry; PUNLMP, papillary urothelial neoplasm of low malignant potential.

IHC of TP53 was performed in 111 early-onset and all consecutive patients. The intensity was recorded as negative in 112, weak in 52 and strong in 60 cases. Similar percentages of positive cells were obtained in the different cohorts (data not shown). A >10% strong staining of TP53 was observed in 29 of the early-onset cases and was not significantly

different to the 23 stained consecutive samples (p=0.39). When we distinguished the tumours according to grade/stage, we detected 14 (22.6%) out of 62 early-onset papillomas, PUNLMPs and pTa low-grade tumours with >10% strong staining. In contrast, only 4 (5.5%) out of 73 consecutive samples of the same grade/stage characteristics were stained (p=0.005). Remarkably, when we also considered a >10% weak staining, 66 (59.4%) early-onset cases showed a >10% weak or strong nuclear accumulation of TP53, whereas only 32 (28.3%) consecutive tumours presenting both intensity categories were included (p<0.0001).

LOH of chromosome 17 and mutations of the *TP53* gene

The analysis of LOH of chromosome 17 and the *TP53* gene did not reveal any significant difference between the early-onset and unselected group (Table 3). All five *TP53* mutations of the early-onset cases were transitions (G:C→A:T; A:T→G:C) and all but one (silent mutation) were miss-sense mutations in the exons 8, 6 and 5. Two mutation carriers were >10% strongly stained for TP53 (Figure 2). Furthermore, ten mutations were discovered in seven (6.2%) consecutive patients showing a >10% strong IHC. Nine transitions (G:C→A:T; C:T→T:A) and one transversion (G:A→C:T) were identified. Seven mutations in exon 8 led to a change in the corresponding amino-acid. One non-sense and two miss-sense mutations were detected in exons 6, 7 and 9.

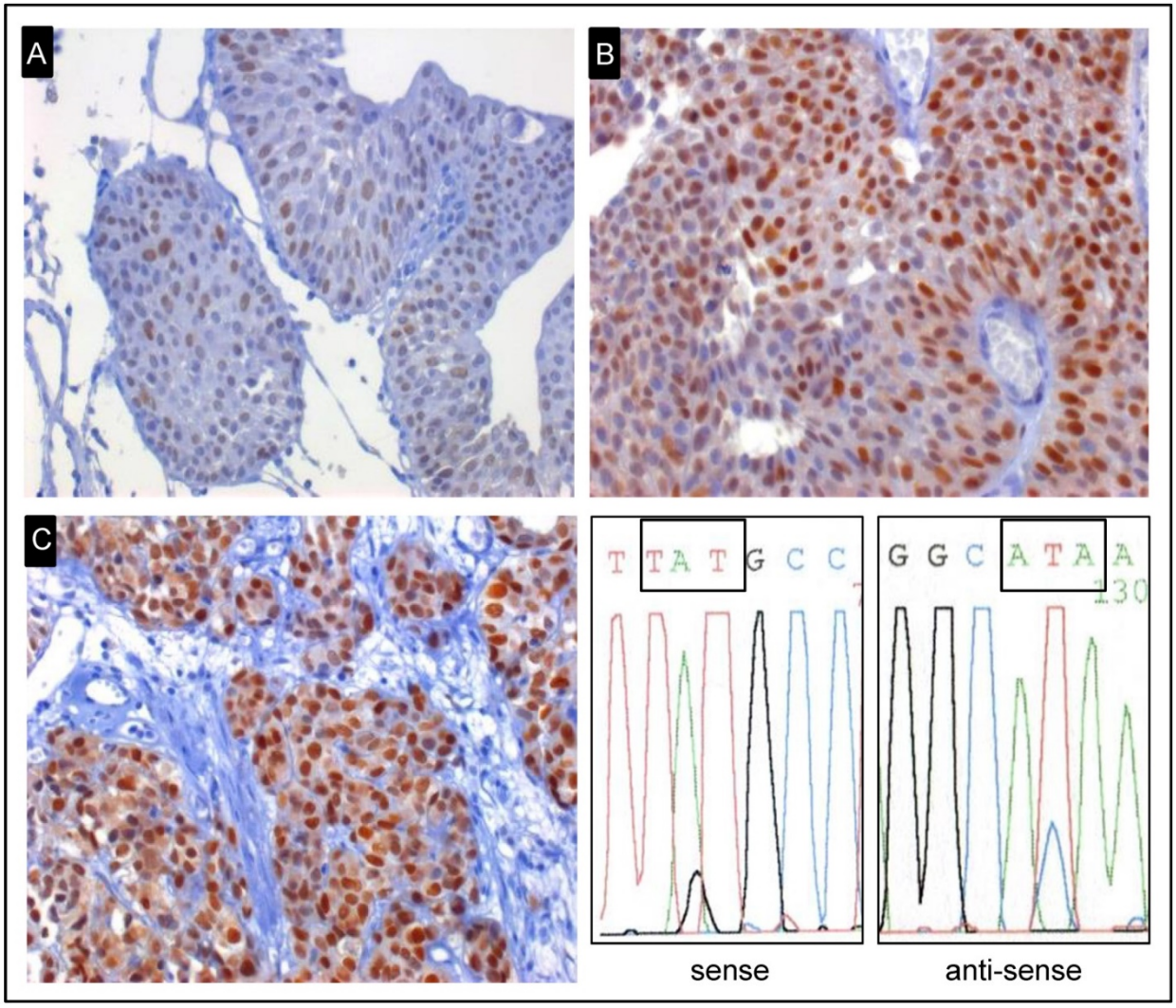

Figure 2: Representative examples of early-onset tumours for TP53 immunohistochemical staining. A, pTa low-grade tumour with a weak expression of TP53 and no *TP53* mutation (x400). B, pTa low-grade tumour with strong expression of TP53 and not mutated (x400). C, Strong expression of TP53 and a mutation in exon 8 were identified in pT2 high-grade tumour (x400). Representative example of sequence analysis of the shown mutated early-onset tumour. The mutation p.C275Y (TGT → TAT) was detected by using Sanger sequencing.

Table 3. Molecular changes of the studied cohorts.

	Early-onset group		Consecutive group		Group comparison
	n	(%)	n	(%)	*p*-value
LOH of chromosome 17					
No	63	(92.6)	62	(91.2)	*p*=1.0
Yes	5	(7.4)	6	(8.8)	
Not informative	28		42		
Not available	22		3		
***TP53* gene**					
Wild-type	100	(95.2)	106	(93.8)	*p*=0.87
Mutated	5	(4.8)	7	(6.2)	
Not available	13		0		
LOH of chromosome 9					
No	67	(70.5)	46	(55.4)	*p*=0.053
Yes	28	(29.5)	37	(44.6)	
9p	6	(6.3)	4	(4.8)	
9q	13	(13.7)	24	(28.9)	
9p+q	9	(9.5)	9	(10.9)	
Not informative	9		30		
Not available	14		0		
***FGFR3* gene**					
Wild-type	74	(65.5)	41	(36.3)	*p*<0.0001
Mutation	39	(34.5)	72	(63.7)	
p.S249C	24	(21.3)	40	(35.4)	
p.Y375C	11	(9.7)	15	(13.3)	
Other mutations	4	(3.5)	17	(15.0)	
Not available	5		0		

LOH; Loss of heterozygosity;

LOH of chromosome 9 and *FGFR3* mutation analysis

A borderline significantly lower percentage of deletions of chromosome 9 was found among the early-onset (29.5%) compared to the consecutive series (44.6%) (p=0.053, Table 3). Moreover, the distribution of chromosome 9 alterations (9p+q vs 9p vs 9q) was not different between the two cohorts (p=0.31). *FGFR3* analysis revealed mutations in 39 (34.5%) of the 113 analysed early-onset tumours (Table 3). Among the unselected cohort, a significantly higher number of 72 (63.7%) patients were detected as mutated (p<0.0001). In both cohorts we identified p.S249C and p.Y375C mutations as the most frequent ones and no difference of the distribution was observed (p=0.24). The frequency of *FGFR3* mutations in different age categories of early-onset patients are shown in Supplementary table 1. Due to low numbers of patients <30 years, we used the median age of 39 to stratify the early-onset cohort. A significantly lower frequency of 12 (23.5%) out of 51 patients aged <39 were mutated compared to 27 (43.5%) out of 62 early-onset patients ≥39 (p=0.04). In contrast, by using the median of 70 years for the consecutive cohort no difference of the distribution was observed (p=0.39, data not shown).

Summarized, any of the typical alterations of UBC were detected in 88 (77.9%) out of 113 analysed consecutive cases, whereas only half of the early-onset tumours were identified as mutated (p<0.0001, Supplementary figure 1). Mutations in *FGFR3* and *TP53* were mutually exclusive, only one consecutive patient presented with a simultaneous mutation.

Discussion

Only few studies have attempted to investigate the biological behaviour of early-onset tumours, with no clear definition regarding the maximum age of onset [9]. Some studies showed a better clinical outcome, especially for patients of the first two decades presenting low-grade and -stage tumours [9, 14, 23]. In contrast, other studies did not observe any difference at the clinicopathological level in the various groups of onset [24]. In other tumour entities the threshold between early-onset and consecutive cancer is mostly defined in the middle years. Early-onset colorectal cancer is mainly reported for patients younger than 50, with the average age of diagnosis of about 70 years [25, 26]. Compared to elderly colorectal cancer, some risk factors and molecular features differ in young patients [27]. As the peak of UBC presents in the seventh decade of life and due to low incidence rates below age 35, we included patients 45 years old or younger. In our study, the early-onset and the unselected group did not show any difference in the grade/stage distribution. The molecular features of young UBC are explored even less (summarized in Table 4).

In elderly patients, mutations of *TP53* occur commonly in muscle invasive tumours and display a key feature in the evolution of carcinoma in situ to an invasive disease [28]. In this study, we did not observe a difference of TP53 nuclear accumulation among the two cohorts using a >10% strong nuclear staining pattern as cut-off. Interestingly, when we compared these strong positive samples in correlation to grade/stage, we detected a distinct distribution among the two series. A significantly higher number of TP53 strongly positive papillomas, PUNLMPS and pTa low-grade tumours were found in the early-onset group. Our results confirmed a previous study by Linn et al. describing 73 patients aged 30 years old or younger, where 67% of early-onset tumours showed a high expression of TP53 by using immunohisto-chemistry [13]. In contrast to our study the intensity of the TP53 staining was not considered. Since almost all tumour samples were non-muscle invasive, the finding of a frequent TP53 overexpression was an unexpected result. When we also included a >10% weak together with a strong IHC staining, we detected as well a significantly higher frequency of positive cells among the early-onset cases.

Table 4. Summary of the different molecular studies of early-onset patients.

Author	Weyerer 2017	Huang 2015	Williamson 2014	Wild 2007	Migaldi 2004	Linn 1998
	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
Number of early-onset patients	118	45	17	14	50	73
Age, range (yr)	17-45	≤30	6-26	4-19	<45	<30
Abnormal expression of CK20[a]	62/99 (62.6)	-	-	2/13 (15.4)	-	-
High expression of Mib1/Ki-67	44/107 (41.1)[b]	-	-	2/13 (15.4)[b]	22/50 (44)[c]	-
TP53 positive staining	29/111 (26.1)[d]	-	-	1/13 (7.7)[b]	28/50 (56)[e]	49/73 (67)[b]
TP53 mutation	5/105 (4.8)[f]	-	0/17[g]	2/12 (16.7)[f]	-	-
Deletion of chromosome 17	5/68 (7.4)[h]	-	1/17 (5.9)[i] Gain	0/4[h]	-	15/72 (20.8)[i]
Deletion of chromosome 9	28/95 (29.5)[j]	-	3/17 (17.7)[k]	0/6[j]	-	9/67 (13.4)[k]
FGFR3 mutation	39/113 (34.5)[l]	13/39 (33.3)[m]	0/17[n]	0/10[l]	-	-

[a] In both studies 10% of stained cells were used as cut-off.
[b] Cut-off level was defined at 10%.
[c] Cut-off level was defined at 15%.
[d] Cut-off level was defined at 10% and the intensity was considered.
[e] Cut-off level was defined at 20%.
[f] Exons 5 to 9 were analysed.
[g] Exons 5, 7 and 8 were analysed.
[h] Marker at chromosome 17p.13.1 (p53Alu) was used.
[i] Chromosome enumeration probe/ centromeric probe for chromosome 17 was used.
[j] Two microsatellite markers at chromosomal region 9p21 (D9S304, D9S1751) and two markers at chromosome arm 9q (D9S303, D9S747) were used.
[k] Locus specific indicator probe for 9p21/ centromeric probe was used.
[l] Nine common mutations were tested by SNaPshot method.
[m] Immunohistochemistry was used for detection.
[n] Exons 7, 10 and 15 were analysed.

By performing molecular analysis of *TP53* we identified a similar percentage of mutations in both cohorts. As it has been shown that a strong staining correlates with *TP53* mutations [29], we did not identify a >10% weak sample as mutated. In a study of atypical bronchial epithelium the lack of *TP53* mutation accompanied with high expressed TP53 without considering the intensity was reported [30]. Due to the absence of mutations the authors suggested that the stained TP53 might be wild-type and might act as a protector against premalignant lesions. Considering both intensity categories and the very frequent staining in both early-onset studies, it appears that wild-type TP53 was identified and that young samples might also try to activate molecular protection mechanisms. Of course there might also be other mechanisms through which the TP53 staining may occur among young cases. For example, the accumulation might as well be due to an inactivation of degeneration pathways [31]. In summary, our results support the hypothesis that TP53 positivity and the distribution according to grade/stage subphenotypes may be dependent from age of disease onset.

Deletions of chromosome 9 are frequent numerical abnormalities in UBC, do not depend on grade/stage distribution and are recognized as one of the initial steps in UBC [6, 7]. LOH of chromosome 9 was only found in 29.5% of early-onset UBC, whereas tumours of the consecutive series showed numerical abnormalities with a frequency of 44.6%. This finding is in line with the two studies of Wild et al. and Williamson et al. in which the authors found infrequent alterations of chromosome 9 [10, 11]. These similar results suggest that early-onset UBC may be more genomically stable.

Activating mutations of *FGFR3* are associated with low-grade non-muscle invasive UBC, with approximately 70% of these tumours carrying alterations [4]. In two recently published studies of urothelial neoplasms in patients <26 years, the total lack of *FGFR3* mutations was reported [10, 11], although almost all tumours presented with a low-grade/stage. An interesting observation in our analysis is that within the early-onset series patients <39 were significantly and infrequently mutated. Therefore, it seems that the difference between early- and the regular-onset of the disease is most pronounced for very young patients. Huang et al. have used IHC of FGFR3 and have reported a frequency of 33.3% of positive stained cells among 45 patients ≤30. Due to clinical differences and since only 13.3% of patients aged 25 or younger were positively stained, they suggested that 25 may represent the cut-off level between early- and regular-onset UBC [12]. Compared to our study, a much lower sample size of early-onset cases was used and the *FGFR3* status was not evaluated by molecular analysis. The authors did not compare the clinical behaviour and staining pattern with consecutive patients, thus they might miss part of the early-onset cases. Summarized, we do not think that 25 may indicate the cut-off for early-onset UBC. The low frequency of *FGFR3* mutations in young cases adds to the evidence, that

mutation profiles and the pathways of carcinogenesis of elderly patients may be less important and distinct molecular features may be involved in the development of early-onset UBC.

Taken together, in only half of the analysed early-onset tumours a typical molecular alteration of bladder cancer could be detected. In two recent studies of BC in young patients below the age of 20 a specific *HRAS* mutation associated with Costello syndrome was found as well as methylation of the *PCDH7* gene in these and older BC patients, suggesting that this may represent a very early change [32-34]. In addition, it was recently shown that activating mutations in the core promoter of the *TERT* gene were detected more infrequently in early-onset compared to consecutive cases [35]. Thus, these results add to a different mechanism and more investigations in early-onset patients are needed to reveal the distinct molecular profile and to better characterize these specific patients.

In conclusion, we demonstrated in the largest cohort of early-onset cases studied, that the known molecular characteristics of UBC are differentially represented in this specific group. In comparison to patients younger or older than 45 years, we observed an infrequent number of *FGFR3* mutations and chromosome 9 deletions and a distinct distribution of TP53 positivity according to grade/stage among young cases. Our data support the hypothesis that the typical profile of UBC is not independent of the age of disease onset and suggest that early-onset tumours represent a differentially biologically driven entity.

Supplementary Material

Supplementary figure 1 and table 1.
http://www.jcancer.org/v08p0323s1.pdf

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Competing Interests

The authors have declared that no competing interest exists.

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