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Original Contributions

Immunohistochemistry in the workup of bladder biopsies: Frequency, variation and utility of use at an academic center

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ABSTRACT

Introduction: Flat urothelial lesions fall into one of four diagnostic categories including urothelial carcinoma in-situ (CIS). There is morphologic overlap between the categories leading to immunohistochemistry (IHC) utilization in difficult cases. The purpose of this study was to examine the frequency, variation and utility of IHC use in bladder biopsy specimens over a 17 year period.

Methods: A search of "CD44", "p53", and "CK20" keywords was conducted from the pathology files (1/1/2003 to 12/31/2017) on bladder biopsy specimens at our institution. Atypical (AUS), dysplastic (UD) and CIS rates were calculated.

Results: A total of 4597 cases were identified. IHC was performed on 345 specimens (7.5%, 345/4597). For cases without IHC (H&E only), the AUS rate was 4.8% (206/4252), UD rate was 9.4% (399/4252), and the CIS rate was 8.4% (359/4252). For IHC cases, the AUS rate was 5.2% (18/345), the UD rate was 8.1% (28/345), and the CIS rate was 11.3% (39/345). There was no statistical difference between the H&E only or IHC rates ($p > 0.05$). The absolute number IHC orders per year increased until 2011 (60 cases) but drastically declined over the last five years (5 total cases in 2017). The CIS rates have remained relatively constant.

Conclusion: We found the AUS, UD and CIS rates were similar regardless of IHC use. Our institution was an early adopter of IHC and it quickly fell out of favor. We agree with the ISUP in that IHC has limited clinical utility for flat urothelial lesions and morphology remains the gold standard.

1. Introduction

Urothelial carcinoma in-situ (CIS) is a precursor lesion to invasive urothelial carcinoma [1,2]. Bladder cancer, among males, is the 4th leading cause of new cancer diagnoses, and it is the 8th cause of cancer related deaths in the US with an estimate of 12,945 deaths in 2018 alone [3]. Even as a precursor lesion, up to 39% of patients with CIS will die from their disease [4]. Furthermore, if left untreated, CIS can become a major cause of morbidity because when it progresses into muscle invasive disease, the patient will require a radical cystectomy [5,6]. CIS is often diagnostically challenging because many of the features seen in malignancy resemble those in reactive atypia [7,8]. Recognizing malignant atypia is crucial to prevent disease progression [9-11].

The International Society of Urological Pathology (ISUP) and World Health Organization (WHO) categorize flat urothelial lesions into four diagnostic categories: hyperplasia, atypia of unknown significance (AUS), urothelial dysplasia (UD) and urothelial carcinoma in-situ (CIS) [12]. However, differentiating these diagnostic categories is difficult and is associated with poor interobserver variability [13,14]. In recent years, there have been several efforts to differentiate atypia based on immunohistochemical staining patterns. Many antibodies have been attempted, but anti-CD20, -CD44 and -p53 have reported the best results [15-18]. Since none of these antibodies alone has strong enough specificity or sensitivity to warrant single antibody usage, an antibody cocktail, composed of all three, has been developed and is commercially available. The name of this cocktail is URO-3 and it initially showed promise [19]. However, upon further review, most of the validation studies for all three antibodies were performed on unequivocally benign or malignant specimens [15-18,20,21]. Thus, the utility of IHC in clinical practice, where morphologically equivocal specimens are commonplace, is unknown. In fact, a recent publication by Arias-Stella et al. showed that in a cohort composed entirely of equivocal specimens, the staining patterns of CK20 and p53 lead to an indeterminant or discoordinate diagnosis 71% of the time [22].

The purpose of this study is to examine the frequency, variation and

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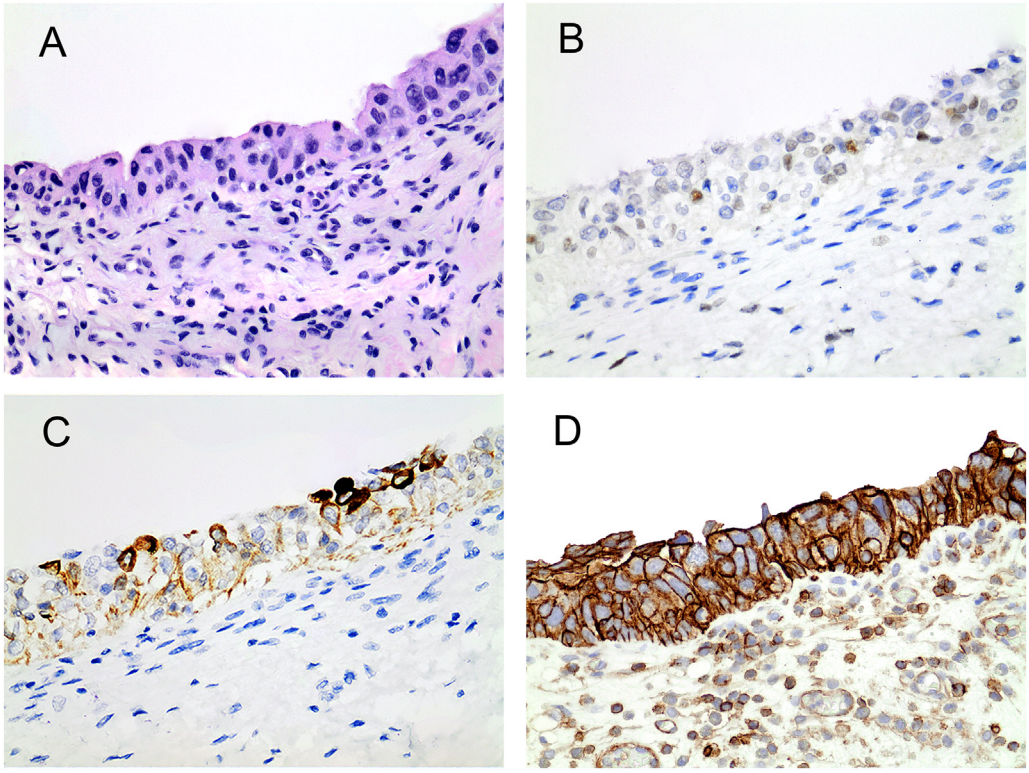

Fig. 1. Benign urothelium immunohistochemical staining pattern. (A) Benign, reactive urothelium. (B) The urothelium is negative for p53 stains and (C) CK20 and is positive for with (D) CD44. Note the CK20 preferentially highlights the superficial cells (C). (Hematoxylin and Eosin, original magnification A ×400, Immunostain, original magnification B–D ×400).

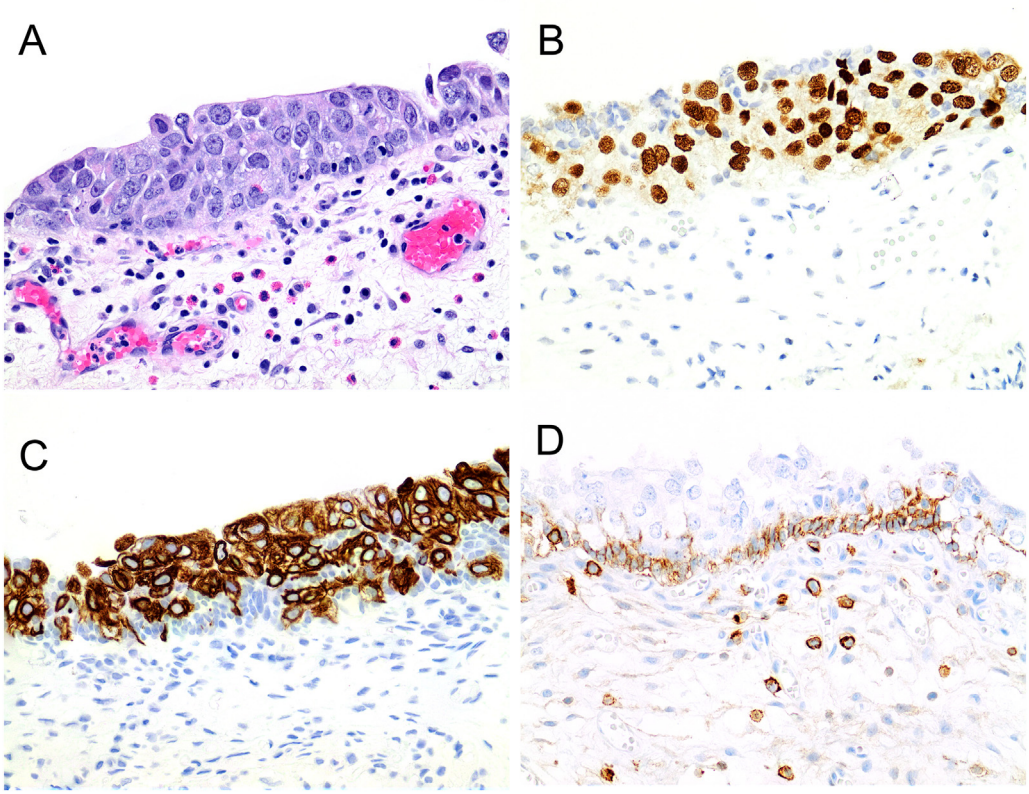

Fig. 2. Urothelial carcinoma in-situ immunohistochemical staining pattern. (A) Urothelial carcinoma in-situ. (B) The carcinoma stains positively with p53, (C) CK20 and negatively with (D) CD44. Note the CD44 immunostain highlights the basal and parabasal cells which are negative for CD20 (C) (Hematoxylin and Eosin, original magnification A ×400, Immunostain, original magnification B–D ×400).

utility of immunohistochemistry (IHC) use in bladder biopsy specimens at an academic institution.

2. Materials and methods

A retrospective search of the pathology files (Sunquest CoPathPlus™ v.6.0.0041, Sunquest Information Systems, Tucson, AZ) from 1/1/2003 to 12/31/2017 was conducted at Loyola University Medical Center. The search included keywords “CD44”, “p53”, and “CK20”. Only flat lesions in surgical biopsy specimens were included. Papillary lesions, invasive carcinomas, transurethral resections and cystectomies were excluded.

Our institution is an academic tertiary care hospital. All pathologists

Table 1
Atypical, dysplastic and carcinoma in-situ rates split by IHC.

Variable	IHC	H&E only	*p*-Value	Totals
AUS	5.2% (18/345)	4.8% (206/4252)	0.7	4.9% (224/4597)
UD	8.1% (28/345)	9.4% (399/4252)	0.5	9.3% (427/4597)
CIS	11.3% (39/345)	8.4% (359/4252)	0.07	8.7% (398/4597)
AUS or greater	24.6% (85/345)	22.7% (964/4252)	0.4	22.9% (1049/4597)
Total cases	7.5% (345/4597)	92.5% (4252/4597)	N/A	N/A

IHC: Immunohistochemistry, H&E: Hematoxylin and Eosin, AUS: Atypia of Undetermined Significance, UD: Urothelial Dysplasia, CIS: Carcinoma in-situ, AUS or greater: rate including all AUS, US and CIS cases, N/A: Not Applicable.

who signed out at least 50 bladder biopsies during the study period were included, ranging in experience from 1 to 25 years. All reports were reviewed and the following parameters were recorded: patient's gender, age, and biopsy location. All diagnoses made after the use of the immunohistochemical stains were recorded for each sample. For the purpose of this study, we defined all biopsies obtained during a biopsy procedure as a single case. Based on the most severe diagnosis made in the case, biopsy results were grouped into the ISUP/WHO diagnostic categories: reactive/benign, atypia of undetermined significance (AUS), urothelial dysplasia (UD) or urothelial carcinoma in-situ (CIS).

All bladder biopsies were performed as part of cystoscopy. The samples were placed into a vial containing 10% buffered formalin solution and labeled with at least two patient identifiers. The specimens were processed using standardized protocols including embedding in paraffin and sectioning at 4–5 μ intervals. The sections were mounted onto charged glass slides and stained with hematoxylin and eosin.

Immunohistochemistry (IHC) was performed on 4 μ thick sections prepared from surgical biopsy specimens. IHC stains were performed at our IHC laboratory on the Ventana Benchmark XT automated immunostaining platform (Ventana Medical Systems, Inc. Tucson, AZ). Prior to staining, the slides were deparaffinized in sequential baths of xylene, transferred to sequential baths of 100% ethanol, followed by sequential baths of 95% ethanol and then rinsed in deionized (DI) water. IHC was performed using mouse anti-human monoclonal anti-CK20 antibody (Ks20.8, Dilution: RTU, Leica Biosystems, Cat# PA0022; Buffalo Grove, IL, USA), anti-p53 antibody (DO-7, Dilution: RTU, Leica Biosystems, Cat# PA0057; Buffalo Grove, IL, USA), and anti-CD44 antibody (SP37, Dilution: RTU, Ventana Medical Systems, 790–4537; Tucson, AZ, USA). The IHC staining pattern for reactive urothelium (Fig. 1) and CIS (Fig. 2) as described by ISUP with p53, CK20 and CD44 immunostains are shown [12]. Institution review board approval was obtained for all aspects of this study.

Descriptive statistics, as well as Fischer's exact tests, were calculated using Microsoft Excel for Mac 2011, version 14.4.2 and www.graphpad.com. All tests were 2-sided and *p*-values of < 0.05 were considered significant.

3. Results

3.1. Patient cohort

A total of 4597 cases were identified during the study period. IHC was performed on 345 surgical biopsy specimens (7.5%, 345/4597). Of the cases in which IHC was performed, the majority of the patients were men (73.9%) with a mean age of 70.8 years (range: 27–96). The remaining women (26.1%) had an average age of 67.5 years (range: 38–91). Of the 4597 biopsy specimens, 1049 (22.9%) were defined as WHO category AUS or greater (including UD and CIS).

3.2. AUS, UD and CIS rates on H&E only

There were 4597 total cases during the study period, of which, 4252 cases (92.5%) were diagnosed on H&E alone. For the H&E only cases, the AUS rate was 4.8% (206/4252), UD rate was 9.4% (399/4252), and the CIS rate was 8.4% (359/4252) (Table 1). The AUS or greater rate (including UD and CIS) for H&E alone was 22.7% (964/4252) (Table 1).

3.3. AUS, UD and CIS rates after IHC

There were 4597 total cases during the study period, of which, 345 cases (7.5%) had ancillary IHC. For the IHC cases, the AUS rate was 5.2% (18/345), the UD rate was 8.1% (28/345), and the CIS rate was 11.3% (39/345) (Table 1). The AUS or greater rate (including UD and CIS) for IHC was 24.6% (85/345) (Table 1). There was no statistical difference calculated between any of the H&E only or IHC rates (Table 1).

3.4. IHC and CIS rate per year

Please see Fig. 3 for a summary of absolute IHC orders and CIS rates per calendar year. The absolute number IHC orders increased yearly until it reached its peak in years 2011 (60 cases) and 2012 (55 cases) (Fig. 3A). The absolute number of IHC orders increased most significantly in year 2011 (Fig. 3A). However, in the subsequent years (2013–2017), the number absolute IHC orders fell to just 10 cases in year 2016 and 5 cases in year 2017 (Fig. 3A). While the percent of cases with IHC declined significantly, the CIS rates remained relatively constant (Fig. 3B).

4. Discussion

The purpose of this study was to examine the frequency, variation and utility of immunohistochemistry (IHC) use in bladder biopsy specimens over a 15 year period at an academic institution. We hypothesized that the atypical, dysplastic and CIS diagnostic category rates would not differ based on if diagnoses were made utilization of IHC or on H&E alone. We found this to be the case and, not surprisingly, there was a drastic decline in IHC order rates over the last five years.

Prior studies have demonstrated challenges for diagnosing flat urothelial lesions despite the ISUP and the WHO framework [12]. In 2010, Isfoss et al. recruited three pathologist to review 81 consecutive biopsies [13] and found there were discrepancies in 41 (52%) of the specimens and the two general pathologists made diagnoses in agreement with the revised final diagnoses in just 56% and 69% of the cases. When compared to the expert/genitourinary (GU) trained pathologists, there were discrepancies in 40% of the cases. More recently, Lawless et al. reviewed 127H&E stained sections of bladder biopsies by 3 pathologists [14]. The overall diagnostic agreement by all 3 pathologists was only 50% with a mean interobserver agreement between any 2 pathologists of 62% (kappa = 0.47). This held true with an expert GU trained pathologist who showed agreement in only 63% (kappa = 0.46) of the cases.

Based on these results, immunohistochemistry (IHC) for differentiating flat urothelial lesions in bladder biopsy specimens was attempted. The typical IHC pattern for CIS, as described by ISUP, is positive staining for CK20 and p53 and negative staining for CD44 (Fig. 2) [12]. However, depending on the case, the IHC results can vary. For example, Yildiz et al. showed that CK20 was diffusely positive in most

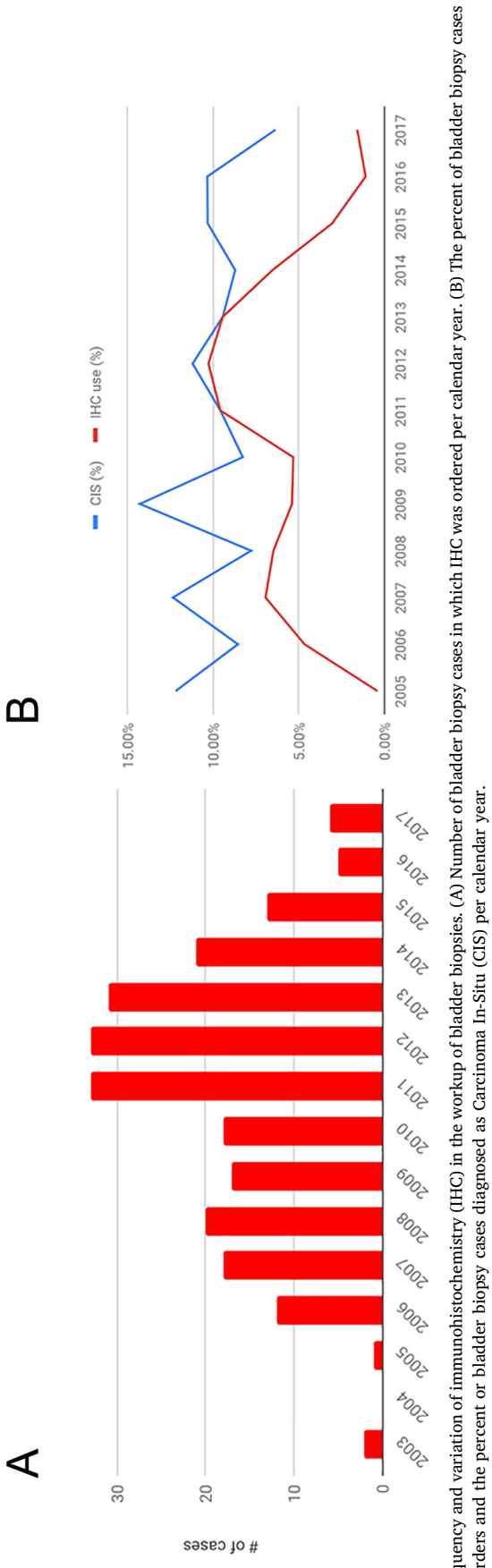

Fig. 3. Frequency and variation of immunohistochemistry (IHC) in the workup of bladder biopsies. (A) Number of bladder biopsy cases in which IHC was ordered per calendar year. (B) The percent of bladder biopsy cases with IHC orders and the percent or bladder biopsy cases diagnosed as Carcinoma In-Situ (CIS) per calendar year.

(89%) CIS cases and the majority were also positive for p53 (62%) [21]. That means, in at least 27% of cases, there were discrepant results between CK20 and p53 staining. Also, Mallofre et al. examined a comparatively larger CIS cohort (50 cases) and found lower rates of CK20 positivity (72%) [15]. The most recent study, conducted by Asgari et al., showed that CK20 was positive in 75% of cases and p53 was positive in 60% of the cases [20]. They also noted that in up to 10% of the cases, discrepancies in CD44 staining (false positives) were found [20].

As for the sensitivity of the IHC markers, McKenney et al. showed that CK20 and p53 IHC had sensitivities of 81% and 57%, respectively [16]. In their study, they found that there was at least one positive immunomarker abnormality in all the examined cases of CIS [16]. However, more recently, Jung et al. examined CIS via tissue microarray and found lower sensitivities for CK20 (61%) and p53 (46%) than previously described [23].

Since each marker shows varying levels of immunoreactivity in CIS, the goal of the URO-3 panel was to combine these markers into a single test for better results. Russo et al. found the URO-3 panel to be inexpensive and useful for differentiating between benign and malignant flat urothelial lesions [19]. More recently, Aron et al. found that URO-3 detected 83% of untreated CIS cases and 71% of treated CIS [24].

While initially URO-3 showed promise, Amin et al., in best practice recommendations from ISUP, states that morphology remains the gold standard for differentiating CIS from reactive atypia [25]. ISUP claims that URO-3 has potential utility but due to variable usage and difficult interpretation patterns, an overreliance on IHC can be misleading [25]. They especially warned against IHC usage in the posttreatment setting and stated that IHC alone cannot definitively differentiate CIS from reactive atypia [25]. Lawless et al. attempted to categorize the staining patters of CK20, p53 and CD44 IHC into four diagnostic categories that mirrored those of ISUP and WHO [14]. They then compared the IHC results with the revised H&E diagnoses (gold standard) and found a pairwise agreement between GU pathologist 1 and GU pathologist 2 in 65.3% (kappa = 0.43) and 57.1% (kappa = 0.40) of cases, respectively [14]. For the general pathologist, there was a pairwise agreement between H&E diagnosis and IHC diagnosis in just 46.9% (kappa = 0.31) of cases [14].

The discrepancy between the initial IHC validation studies and the lack of clinical utility of URO-3 remains a mystery. One explanation is that prior to 2017, IHC validation studies came from unequivocally benign or malignant specimens. In practice, cases requiring adjuvant IHC studies rarely show ideal histology. Furthermore, many of these cases contain artifact including poor tissue orientation, small specimens and fragmentation. To address these concerns, Arias-Stella et al. created a small cohort comprised of entirely equivocal diagnoses [22]. Of the 69 cases with equivocal diagnoses, 71% were either discordant or indeterminant CK20 or p53 staining patterns while only 9 cases showed the ISUP CIS staining pattern [22]. While there may be some clinical utility for IHC in flat bladder lesions, the results from cases with equivocal diagnoses are less impressive and of more clinical significance.

In the present study we found a similar atypical, dysplastic and CIS rates regardless if IHC was performed or not. While none of the rates reached statistical significance, the closest was the CIS rate ($p = 0.07$). This is likely due to a selection bias, as pathologists were probably more likely to perform IHC on cases that were suspicious for CIS. If so, the IHC was used for confirmation rather than for diagnosis. It is likely that many of these IHC cases could have been diagnosed on morphology alone. Another finding in this study was that we saw our pathologists were early adopters of IHC, peaking in years 2011 (60 cases/year) and 2012 (55 cases/year). However, IHC quickly fell out of favor to a mere 5 cases in year 2017.

The major limitation of this study is the retrospective nature of the design. It is difficult to assess the appropriateness of the IHC use for a given case. Possible uses of IHC include indeterminate morphology,

diagnosis confirmation and for teaching purposes. If a large portion of the IHC was inappropriately ordered (i.e. for any reason other than 'indeterminate morphology'), then it would not be surprising that the atypical, dysplastic and CIS rates were similar. However, this is likely not the case as we saw a drastic decline in IHC order rates, especially over the last five years. If the IHC was useful for differentiating the diagnostic categories, we would have expected the IHC orders to increase.

While immunohistochemistry for flat bladder lesions initially showed promise, we found the atypical, dysplastic and CIS rates were similar regardless of IHC use. Additionally, our institution was an early adopter of IHC but it quickly fell out of favor to a total of only 5 cases in 2017. Therefore, we agree with ISUP in that IHC has limited clinical utility and morphology remains the gold standard [25].

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