Original Research Article

DETERMINING THE ROLE OF IMMUNOHISTOCHEMICAL EXPRESSION OF CK20 IN GRADING UROTHELIAL CARCINOMA

ABSTRACT:

Background: The cytoskeleton-associated intermediate filaments, CK20 belongs to epithelial subgroups and it has a controlled expression in gastrointestinal marked cells and in the urinary tract. During embryogenesis, cytokeratin is the first intermediate filament type and they provide facilitation in maintaining the integrity and mechanical stability of cells. It is identified that neoplastic urothelial cells there is strong and diffuse cytoplasmic immunoreactivity for CK20 that involve all three layers of the urothelium therefore the aim of the study was to determine the role of immunohistochemical expression of CK20 in grading urothelial carcinoma.

Methodology: It was a retrospective cross-sectional study conducted at the Department of Histopathology, Lady Reading Hospital, Peshawar, Pathology department, Sheikh Zayed medical college & Hospital, Rahimyar Khan and Khyber Medical college & Khyber teaching Hospital, Peshawar from March till September 2021. 97 Paraffin embedded blocks of urothelial carcinoma along with record of these patients, collected over three years from January 2018 till December 2020, were retrieved from archives of Histopathology departments of these hospitals. These blocks were cut and stained with Hematoxylin and Eosin and CK20 antibody. Expression of CK20 was noted by two consultant pathologists. Positive expression was considered when cytoplasmic immunoexpression of CK20 was seen in deep layers of urothelium or diffused strong cytoplasmic staining of urothelial cells beyond the superficial cells. Negative expression was defined as CK 20 staining restricted to superficial cells. Negative expression was defined as CK 20 staining restricted to superficial "Umbrella" cells of urothelium.

Results: Out of 97 patients, the minimum age was 20 years and maximum age was found to be 90 years with mean $\pm$ standard deviation of 64 $\pm$11 years. There were 86 (88.6%) male patients and 11 (11.3) female patients. Low grade urothelial carcinoma was seen in 49(51 %) patients

while 48 (49%) cases displayed high grade morphology. CK20 was found positive in 74(76.3%) patients. Among positive cases, 43 cases were high grade and 31 cases were low grade urothelial carcinoma.

CONCLUSION: CK20Positivity was seen in76.3% patients with papillary urothelial carcinoma. CK20 is important immunohistochemical marker for early diagnosis and grading of urothelial carcinomas.

KEY WORDS: CK20, urothelial carcinoma, Immunohistochemical expression.

INTRODUCTION

Among the cancers of the urinary system, bladder cancer is the most common malignancy with that affected 549,393 individuals and caused the death of 199,922 patients across the globe in 2018. Histologically, Urothelial carcinoma (UC) accounts for 90% of bladder malignancies among all the reported cases (1). It is estimated that bladder carcinoma is the 9th most common cancer worldwide and the thirteenth most frequent cause of death among the patients with cancers associated to the urinary system (2). In the United States, it is the fourth most common cancer in males and the tenth most common tumor in females with a male to female ratio of 3:1 and the median age of diagnosis is 68years (3). In Pakistan, a study conducted in Armed Forces Institute of Pathology Rawalpindi showed that bladder carcinoma is the 7th most common tumor in both males and females and represented 93.4% of all bladder cancers (2).

It has been identified that cigarette smoking has a strong association with bladder cancer (4). Other predisposing factors include arylamines, aniline dyes, phenacetin, auramine and cyclophosphamide, Schistosoma haematobium, and prior radiation exposure of bladder for treatment of prostatic cancer (5). WHO/ISUP in its latest classification of tumors of the urinary tract classified urothelial tumors into infiltrating and noninvasive urothelial neoplasms. Among the infiltrating Urothelial carcinomas, various subtypes like Nested, Microcystic, Micropapillary, Plasmacytoid, Sarcomatoid, Giant cell, Lymphoepithelioma like Lipid rich, Clear cell and Poorly differentiated, are included. The noninvasive urothelial neoplasms are subclassified into Urothelial carcinoma in situ, papilloma, papillary urothelial neoplasm of low-grade malignant potential (PUNLMP), Noninvasive papillary urothelial carcinoma, low grade(LGUC), and Noninvasive papillary urothelial carcinoma, high-grade (HGUC) (6). Prognosis of urothelial carcinoma is determined by various factors like pathological grade and stage of tumor, muscular propria invasion, and age of the patient (7). Among these, the Grade of the tumor is the single

most important prognostic factor. The prognosis of LGUCis generally good, though these tumors show high Recurrence rates. Approximately 30% of these recurrent tumors progress by invading lamina propria (8).

Among the cytoskeleton-associated intermediate filaments, CK20 belongs to epithelial subgroups and it has a controlled expression in gastrointestinal marked cells and in the urinary tract. During embryogenesis, cytokeratin is the first intermediate filament type and they provide facilitation in maintaining the integrity and mechanical stability of cells. The urothelium of the bladder has multilayered epithelium with three different cell zones i.e. i. basal cells ii. intermediate cells zone and iii. superficial cell layer. In adults, urothelium CK20 expression is restricted to the superficial cell umbrella layer however, in neoplastic urothelial cells there is strong and diffuse cytoplasmic immunoreactivity for CK20 that involve all three layers of the urothelium (9). The staining pattern of CK20 can be an adjunct in early diagnosis of urothelial cancer and can envisage malignant potential in low grade urothelial tumors (10). This study was designed to delineate the diagnostic utility of CK20 antibody in classifying urothelial carcinoma into high grade and low-grade forms based on expression of CK20.

METHODOLOGY

Aretrospective cross-sectional study was conducted at the department of Histopathology, Lady Reading Hospital, Peshawar in collaboration with Pathology department, Sheikh Zayed medical college & Hospital, Rahim Yar khan and Khyber Medical college & teaching Hospital, Peshawar from March till September 2021. Sample size was calculated using WHO sample size calculator according to following parameters; Confidence level (1-a=95 %Anticipated population proportion (P) = 49.5%Absolute precision required (d) = 10 %Minimum sample size (n) = 97. Formalin fixed, Paraffin embedded 97 blocks of already diagnosedcases of urothelial carcinoma were included in the study using non probability, consecutive sampling technique. Poorly preserved, autolyzed and inadequate bladder biopsies were excluded from the study. Data collection proforma of the patients was filled with record retrieved from histopathology data bank of these institutions. Retrieved blocks were cut at 3-5um thick sections for routine Hematoxylin and Eosin staining. Immunohistochemical assay for CK20 were done by using DAKO kit according to manufacturer's guidelines as follow: The blocks were sectioned at 3 um thickness and placed on clean glass slide with pre-attached adhesive on its surface. They were incubated at 58 degrees Celsius for 4 hours. The sections were deparaffinized with xylene 1 and

2, for 3 minutes each. They were rehydrated in decreasing concentrations of alcohol, 90% 80% and 70% for 3 minutes each. The slides were placed in coplin jar with 0.01 M Tris-EDTA buffer at pH of 9.0. 750W domestic microwave was used to treat the slides for 20-30 minutes for heat mediated antigen retrieval.Slides were washed with distilled water for 20-40 minutes. After cooling down the sections, they were brought to phosphate buffered saline (PBS) at pH 7.3 for 5 minutes. PBS was washed and excess was wiped off the sections. Endogenous peroxides activity was blocked by incubation in 0.5% hydrogen peroxide in methanol for 5 minutes. The slides were washed in three series of PBS, 2 minutes each. 100 microliters of primary antibody of CK20were instilled on the sections and incubated for 60 minutes. The slides were again washed in three series of PBS, for 60 minutes. The slides were then incubated in avid in-biotin complex for 10 minutes. They were rinsed with distilled water and then incubated in DAP (diaminobenzidine) substrate solution for 5 minutes. Then the slides were washed with water and counter stained with hematoxylin for 40 seconds. The slides were dehydrated by placing them in increasing concentrations of alcohol 70%,80%,90% and 100% alcohol for 3 minutes each. Clearing was done by placing slides in xylene for 3 minutes. The slides were mounted with Canada balsam. Non neoplastic urinary bladder tissue was used as negative control while formalin fixed paraffin embedded block of skin tissue was used as positive control. Slides were examined by two consultant histopathologists, blind to the original issued reports. Histopathologic diagnoses were made for all the cases included in study and recorded in a predesigned proforma. Then CK20antibody stained slides were examined and each case was assigned positive or negative status based on nuclear staining intensity and percentage score. Strong nuclear immunoreactivity in more than 10% of tumor cells was considered positive. Negative status was recorded for a case with either no staining or staining in less than 10% of tumor cells. Gender, age, CK20 immunohistochemical expression and grade of tumor were recorded. SPSS version 26 was employed for statistical analysis. Calculation of mean and Standard Deviation was done for numerical variables like age. Gender, age, tumor grade and immunohistochemical expression of CK20 were expressed in frequencies and percentages. Through stratification, effect modifiers including age, tumor grade and gender were controlled. T-test was applied by taking p-value of less than 0.05 as significant.

RESULTS

Among 97cases, the minimum and maximum age of patient recorded was 20 years and 90 years with mean± standard deviation as 41.18 ± 12.66 years. There were 86 (88.6%) male patients and 11 (11.3%) patients were females making male to female ratio of 7.8:1. 42 males had HGUC while 44 male patients were diagnosed with LGUC. Among the 11 female patients, 06 cases showed high grade morphology while 05 cases were classified as LGUC. Papillary urothelial carcinoma, low grade (LGUC) was seen in 49 (50.5%) patients whilesections from 48 (49.5%) cases showed papillary urothelial carcinoma, high grade (HGUC) as shown in Figure 1.Immunohistochemically CK20 was found positive in 78 (80.4%) patients and it was negative in 19 (19.6%) patients. Among the positive cases, 46 were HGUC and 32 were LGUC. Out of 19 negative cases, 02 cases showed high grade morphology while 17 cases showed features of LGUC as shown in Table 1.Among the CK20 positive cases, 29 cases showed muscle invasion while muscle invasion was seen in 03 cases which did not show any CK20 expression making a highly significant correlation between muscle invasion and CK20 expression (p-value <0.001) as shown in table 2. Expression of CK20 was seen in 68 male patients and 10 female patients while no expression was seen in 18 male patients and 1 female patient (p value< 0.001) as shown in table 3.

Table 1: Co-relation of CK20 expression with tumor grade

	CK20 Expression				
Tumor Grade	Negative	Frequency (%)	Positive	Frequency (%)	p-value
High Grade	02	02	46	47	P<0.001*
Low Grade	17	18	32	33	
Total	19	19.6	78	80.4	

*p-value is significant at <0.05 level

Table 2: Co-relation of CK20 expression and muscle invasion.

	Muscle Invasion		
CK20 expression	Present	Absent	p-value
Negative	3	16	p<0.001*
Positive	29	49	
Total	**32**	**65**	

*p-value is significant at <0.05 level

Table 3: Co-relation of CK20 expression and patients' gender

Gender	CK20 Expression		p-value
	Negative	Positive	
Male	18	68	p<0.001*
Female	1	10	
Total	**19**	**78**	

.*p-value is significant at <0.05 level

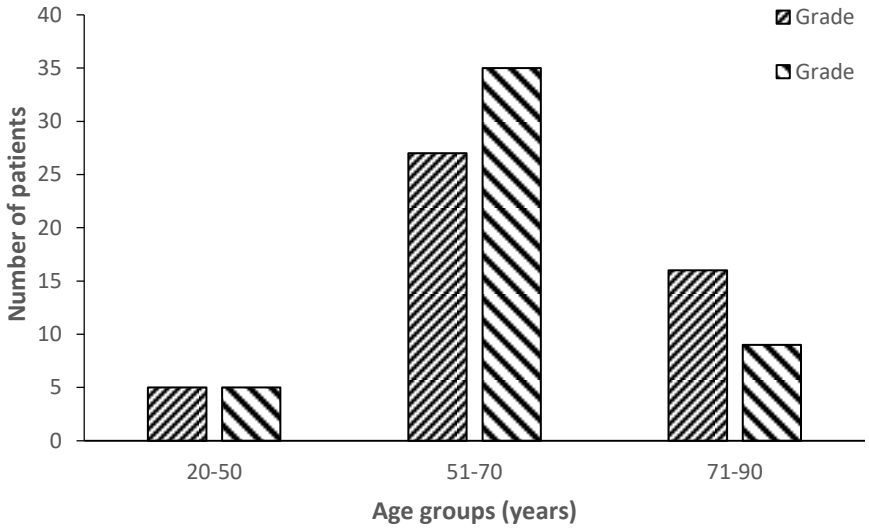

Figure 1: Co-relation of patients' age group with tumor grade.

Table.4: Distribution of Different Grades of Urothelial Carcinoma (n=97)

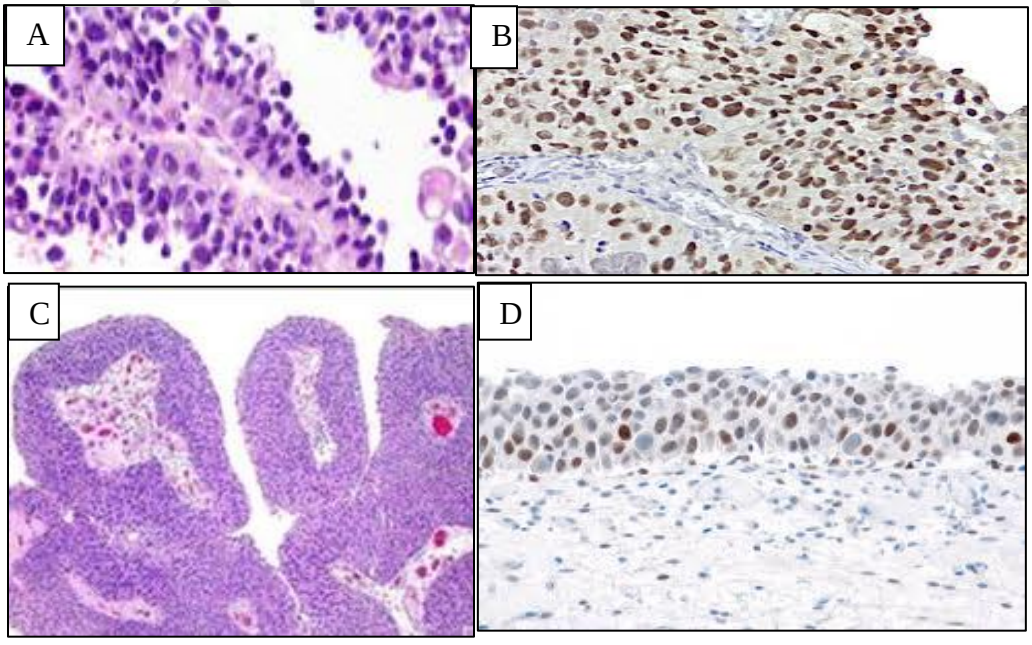

Figure 2. Noninvasive papillary urothelial carcinoma. High grade urothelial carcinoma H&E (A), Strong CK20 (B); Lowgrade urothelial carcinoma H&E (C), Weak CK20 (D).

DISCUSSION

With the advancement in diagnostic methods over the last two decades, it is now common to use immunohistochemical markers for assessing predictive and prognostic potential of various tumors in this regard multiple immunohistochemical biomarkers have been explored in cases of urothelial carcinoma of urinary bladder (11). In current study we assessed the role of CK20in grading of urothelial carcinoma. In present study the role of ck20 in grading of urothelial carcinoma CK20 belongs to family intermediate filaments was assessed. A total of 97 patients were included in our study the calculated mean age was 64$\pm$11 years. There were 86 (88.6%) male patients and 11(11.3%) female patients, making male to female ratio 7.8:1. Opposite to our findings the male to female ratio of 4:1 was published by a group who included the same number of patients in their research (5) however in another study investigators found male to female ratio of 7.46:1 which is concordant with our results (12).We did not find any significant association between age group and grading of urothelial carcinoma. A study was conducted in Turkey over 18 years to see the potential effect of age on the natural behavior and progression of bladder cancer in different age groups. They found that single and small tumors were usually present in patients younger than 40 years with lower recurrence rate but similar tumor progression rate in both young and old age groups. They concluded their manuscript with remarks that invasiveness of tumors in young patient must be assessed carefully, and intervention should be initiated earlier to halt the progression for better outcome(13).

In the present project, 78 (80.4%) of the cases yielded positive results while 19 (19.6%) cases showed negative results for CK20 over expression on application of CK20 antibody. Among

CK20 positive cases, 46 cases were HGUC and 02 cases were LGUC. Among the negative 19 cases, 17 cases were LGUC while 32 cases showed high grade morphology on hematoxylin and Eosin-stained sections. CK20 was positive in 74(76.3%) patients with 43 high grade and 31 low grades. It was negative in 23(23.7%) patients with 5 high grade and 18 low grade carcinomas. Study concluded CK20 in 33 cases (68.8%) useful marker in grading of urothelial cancer where features are borderline (5). Mumtaz et al shared diffuse positive expression of high grade and 16(40%) of low grade tumor.CK 20 expression for grading of urothelial carcinoma has been studied widely done and with other immunohistochemical biomarkers. Yin H et al, studied three immunohistochemical markers p53, CK20 and Ki67 on 84 urothelial tumors using WHO/ISOP classification for grading. They found 64% low grade and all high-grade carcinomas show diffuse staining of CK20 while remaining (13) 36% low grade carcinoma show focal staining. These findings are in accordance with our study.

Double staining of cytokeratin is helpful tool to identify the behavior and nature of urothelial carcinoma. CK20 expression has potential to predict the malignant changes in low grade urothelial carcinoma and the diffused CK20 staining along with loss of CK5/6 has been associated with aggressive behavior of carcinoma (9). In another study it has been suggested by sheikh et al that CK20 expression has a role in assessment of recurrence of low-grade urothelial carcinoma. The tumors with chromal CK20 expression are more likely to reoccur (14).

The objective of current study was to assess the relationship of immunoreactivity of CK20 across the spectrum of urothelial neoplasia using WHO/ISUP classification. The study concluded that increased CK20 positivity was significantly associated with increased tumor grade and stage (15).

Urothelial carcinoma in situ in bladder can be difficult to diagnose due to factors including procedures artifacts, therapy related changes and different growth patterns. In difficult cases CK 20 was more sensitive, consistent strong staining as compared to alpha-methylalcyl-CoA racemase (AMACR) (16). In another study Gata3, CK 20, CK5/6 and CK14 immunohistochemical markers were used for subtyping bladder cancer. Positive expression of CK20, was 50.8% in patients that have muscle invasive bladder cancer. GATA3, CK20, CK5/6, CKU staining were used to predict clinical outcome and survival after radical cystectomy. CK20 immunopositivity expression was noted in luminal molecular subtype (17).

According to this study, urothelial carcinomas are divided into two immunocytochemical subtype. Basal subtype which are CK5 positive, CK20 negative or CK5 positive and GATA3 negative). The luminal subtype is (CK5 negative and CK20 positive) In this study CK20 expression was used in molecular subtyping of urothelial carcinoma (18). In this study, CK20 and CK5/6 IHC panel was used to classify the urothelial carcinoma into 3 groups, it was noted that high expression at CK20 in group3 was associated with worse progress. According to molecular study there is identified cell adhesion and increased proliferation in group 3. Therefore, CK20 can be used as easily accessible prognostic marker in daily practice. As its high expression is associated with worse prognosis (19).

Urinary bladder flat lesion carcinoma in situ is a worrisome lesion, which require aggressive surveillance and treatment, CK20 is most widely used immunohistochemical marker for discrimination of urothelial cis from reign or reactive urothelium. CK20 was positive in 20 specimens (29%), 11 (16%) showed patchy equivocal staining, 38 (55%) had negative staining (20). In another descriptive study, urinary bladder samples were collected from 50 patients in Iran.

According to that study CK20 positivity was 84.6%. They concluded CK20 as specific marker for diagnosis of low and high grade pupillary urothelial carcinoma.

CONCLUSION

Urothelial carcinoma progresses by acquiring mutations, notably mutations in CK20. This mutation has a diagnostic and prognostic value. Immunohistochemical staining for CK20 antibody not just aid in early diagnosis but it also underscores important prognostic connotation. Strong CK20 positivity is seen is seen in cases of HGUC while weak to absent staining is seen in cases of LGUC. Moreover, CK20 negativity is also required in patients with urothelial carcinoma undergoing treatment with bacilli Calmette–Guerin. We recommend using CK20 antibody in cases of urothelial carcinoma since it has a diagnostic and prognostic value.

References:

1. Hepp Z, Shah SN, Smoyer K, Vadagam P. Epidemiology and treatment patterns for locally advanced or metastatic urothelial carcinoma: a systematic literature review and gap

analysis. J Manag Care Spec Pharm. 2021;27(2):240–55.

2. Atique M, Abbasi MS, Jamal S, Khadim MT, Akhtar F, Jamal N. CD 10 Expression Intensity in Various Grades and Stages of Urothelial Carcinoma of Urinary Bladder. J Coll Physicians Surg Pakistan. 2014;24(5):351–5.
3. Babjuk M, Oosterlinck W, Sylvester R, Kaasinen E, Böhle A, Palou-Redorta J, et al. EAU guidelines on non-muscle-invasive urothelial carcinoma of the bladder, the 2011 update. Eur Urol. 2011;59(6):997–1008.
4. Miyazaki J, Nishiyama H. Epidemiology of urothelial carcinoma. Int J Urol. 2017;24(10):730–4.
5. Mumtaz S, Hashmi AA, Hasan SH, Edhi MM, Khan M. Diagnostic utility of CK20 and CK20 immunohistochemical expression grading urothelial malignancies. Int Arch Med. 2014;7(1):1–8.
6. Humphrey PA, Moch H, Cubilla AL, Ulbright TM, Reuter VE. The 2016 WHO Classification of Tumours of the Urinary System and Male Genital Organs—Part B: Prostate and Bladder Tumours. Eur Urol [Internet]. 2016;70(1):106–19. Available from: http://dx.doi.org/10.1016/j.eururo.2016.02.028
7. Vaidya S, Lakhey M, K C S, Hirachand S. Urothelial tumours of the urinary bladder: a histopathological study of cystoscopic biopsies. JNMA J Nepal Med Assoc. 2013;52(191):475–8.
8. Rouprêt M, Hupertan V, Seisen T, Colin P, Xylinas E, Yates DR, et al. Prediction of cancer specific survival after radical nephroureterectomy for upper tract urothelial carcinoma: Development of an optimized postoperative nomogram using decision curve analysis. J Urol [Internet]. 2013;189(5):1662–9. Available from: http://dx.doi.org/10.1016/j.juro.2012.10.057
9. Advance in virology.2020. CK20 and CK5/6 immunohistochemical stain of uritheral neoplasm: A perspective.
10. PLOS one, no.6, Artticle ID e0179602,2017
11. Kardoust Parizi M, Margulis V, Compe´rat E, Shariat SF. The value and limitations of urothelial bladder carcinoma molecular classifications to predict oncological outcomes and cancer treatment response: A systematic review and meta-analysis. Urol Oncol Semin Orig Investig. 2021;39(1):15–33.

12. Thakur B, Kishore S, Dutta K, Kaushik S, Bhardwaj A. Role of CK20 and Ki-67 immunomarkers in carcinoma of urinary bladder. Indian J Pathol Microbiol [Internet]. 2017 Oct 1;60(4):505–9. Available from: https://www.ijpmonline.org/article.asp?issn=0377-4929
13. Gunlusoy B, Ceylan Y, Degirmenci T, Kozacioglu Z, Yonguc T, Bozkurt H, et al. Urothelial bladder cancer in young adults: Diagnosis, treatment and clinical behaviour. J Can Urol Assoc. 2015;9(9-10 October):E727–30.
14. Yin H, LeorjAs: Histologic grading of noninvasive papillary urothelial tumor : Variation of 1998 WHO/ISUP system by immunophenotyping and fellow up. AmJ chin pathol 2004, 121(5): 679-687.
15. Comparison of WHO/ISUP classification and cytokeratin 2. expression in predicting the behaviour of low grade papillary urothelial tumors. A. Alsheikh M.
16. Relationship of CK20 and CD44 protein. expression with WHO/ISUP grade in pTa and Pt1 papillary urothelial neoplasia.
17. Does the addition of AMACR to CK20 help to diagnose challenging case of urothelial carcinoma in situ.
18. Impact of immunohistochemistry basd subtyping of GATA3,CK20, CK5/6 and CK14 expression on survival after radical cystectomy of muscle invasive bladder cancer (2021). Article made 31186.
19. Combine duse of immunohistochemical markers of basal and luminal subtypes in urothelial carcinoma of bladder, association with chincopathological features and outcomes. April 2021.
20. Transcriptiral analysis of immunohistochemically defined subgroups of non muscle invasive papillary high grade upper tract urothelial carcinoma M.Jung 2019.
21. CK20 and P53 immunohistochemical staining pattern in urinary bladder with qeuivocal aypia. Javier A-Nias steua 3rd Archive pathology 2018