

# Utility of Modified PAP Stain in Histopathology for Demonstration of Keratin

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## ABSTRACT

**Introduction:** Keratins are intermediate filament proteins which give structural stability to the cell. In routine Hematoxylin & Eosin stain, they appear as eosinophilic material. A few other stains are described in literature for the demonstration of keratin. The aim of this study was to assess the utility of modified PAP stain in the demonstration of keratin in histopathology sections. **Materials and Methods:** In this study, 89 histopathological cases showing keratin were included. These were comprised of various benign and malignant lesions involving squamous epithelium. Two sections were taken from the paraffin block of each case. H & E stain and modified PAP stain were done in all. These two stains were compared based on 4 parameters: Staining quality, background clarity, morphology and differentiation. Scores were allotted ranging from 1 to 3 for each parameter. They were statistically analysed by 'unpaired t test' at 95% confidence level ( $p \leq 0.05$ ). **Results:** It was found that there was no significant difference in the staining characteristics between two stains, based on the 4 parameters. But individual lesions show variable staining of keratin when the two stains were compared. **Discussion and Conclusion:** A few available similar studies showed a distinct advantage of modified PAP compared to H&E. In our study modified PAP was found useful in only a few conditions like squamous cell carcinomas in which the differentiation or contrast was excellent when compared to H&E stain.

**Key Words:** Keratin, Modified PAP, Hematoxylin & Eosin

## INTRODUCTION

Keratins are intracytoplasmic structural proteins which are the dominant intermediate filament proteins of keratinocytes and hair forming cells. They account for up to 80% of the total cell protein in differentiated keratinocytes. They are water-insoluble. Keratins are obligate heteropolymers and they contain a dimeric central  $\alpha$ -helical rod domain that is flanked by non-helical head and tail domains. The 10-nm keratin filaments participate in the formation a dense proteinaceous structural network within the cellular cytoplasm, radiating from the nucleus to the plasma membrane. It acts as cytoplasmic scaffold that gives the ability to sustain mechanical and non-mechanical stress. Intermediate filaments are highly dynamic structures and are reorganized during mitosis and apoptosis; reorganization is moderated by glycosylation, posttranslational phosphorylation, transglutamination and proteolysis, or through interaction with other proteins

The first nomenclature of keratins was published in 1982 years by Moll et al. [1] The concept of two types of keratin proteins, began to emerge after that. With the use of two-dimensional polyacrylamide gel electrophoresis depending on molecular weight (40 to 68 kDa) and isoelectric pH (5 to 8), the keratins were numbered, based on the location of each protein. Neutral-basic keratins were numbered from largest to smallest, K1-K8, and the acidic keratins

were numbered similarly, K9–K19. The polymorphic variant of K10 that migrated faster became K11. [2] Over 25 subtypes are defined and given Moll catalog number ranges from 1 (highest molecular weight) to 23 (lowest molecular weight) they are divided into Type I (acidic; CK10, CK12-19, 40-56.5 kDa) and Type II (neutral-basic, CK1-CK8, 53-67 kDa). Type I genes are located at 17q21.2, type II genes at 12q13.13.

Since their initial characterization almost 30 years ago, the total number of mammalian keratins has increased to 54, including 28 type I and 26 type II keratins. They play a significant role in epithelial cell protection from mechanical and non-mechanical stressors. This was substantiated by several studies in keratin knockout and transgenic mice. It leads to the discovery that human keratin mutations can predispose to tissue-specific injury. Recently it was found out that keratins play an important role in other cellular functions, including cell size, protein synthesis apico-basal polarization, motility, membrane traffic and signaling. A major turning point came with the discoveries that mutations in keratin intermediate filament genes were responsible for a large number of inherited skin fragility disorders like epidermolysis bullosa simplex. [3] There is widespread use of keratins as diagnostic tumour markers in Immunohistochemistry. Epithelial malignancies (carcinomas) can be differentiated from sarcomas and lymphomas because the epithelial cells show the specific keratin pattern. [4] This is particularly useful in poorly differentiated carcinomas where the epithelial morphology cannot be discerned easily by routine H&E staining.

In histopathology we come across various neoplastic and non neoplastic conditions in which keratin can be seen. Benign keratinocytic lesions include seborrheic keratoses, corns, calluses, epidermal cyst, dermoid cyst, steatocystoma, squamous papillomas, verrucous hyperplasia etc. Hyperkeratosis

can be due to various acquired or genetic conditions. Examples include keratosis pilaris, Plantar hyperkeratosis Epidermolytic hyperkeratosis etc. Kertatoactomas are benign lesions which are close mimickers of squamous cell carcinomas. [4] Identification of Keratin pearl is the defining feature of well differentiated squamous cell carcinomas. In routine hematoxylin and eosin (H-E) staining, keratin has eosinophilic. Other substances like collagen, amyloid, muscle and other extra cellular and intracellular secretions also are eosinophilic. A well trained pathologist can differentiate these based on the minute details. But in some situations it is difficult. Cytochemical stains can play a pivotal role in such situations. Nowadays modern laboratories are using *the immunohistochemical methods for presence of keratin protein by using different types of antikeratin antibody. Immunohistochemical technique in paraffin embedded sections require deparaffinization, antigen retrieval, addition of primary, secondary antibody and substrate, all these steps needs appropriate time for incubation, so this is more time consuming procedure. It is very expensive also.* The aim of this study was to evaluate the efficacy of modified papanicolaou (PAP) stain for demonstration of keratin in histopathology sections.

The Papanicolaou stain (PAP stain) was first developed by father of cytopathology George N Papanicolaou. The Papanicolaou stain uses the polychromatic staining technique and uses different colours to differentiate the cells in gynaecological and nongynaecological samples. It also helps in the analysis of cytological aspects and permits the identification of basic inflammatory, dysplastic or malignant process in non-gynaecological specimen such as fine needle aspiration cytology and other bodily secretions. [6] It stains preferentially based on the degree of cell maturity and cellular metabolic activity. It is designed to meet three staining objectives: - Definition of nuclear details, transparency of cytoplasm and differentiation of cells

Haematoxylin is the nuclear dye in the Papanicolaou procedure, which demonstrates chromatin pattern of normal and abnormal cells. The nuclear stain acts as a mordant, solvent, oxidizing agent, and as a substance that is used for acidification. The cytoplasmic counter stains orange G and eosin azure have a high alcoholic concentration that provides cytoplasmic transparency, which enables clear visualization through overlapped cells, mucous, and debris. However, the Papanicolaou stain is used in tissue sections for better contrast and demonstration of keratin and cells such as epithelial cells, connective tissue, muscles and red blood cells. [6]

Haematoxylin and eosin (H& E) has always been considered the gold standard in staining tissues but sometimes this staining techniques has its limitations, for example, the colour contrast cannot be appreciated at all times, which leads to ambiguity in diagnosis, especially in cases like moderately or poorly differentiated squamous cell carcinoma, where at times, it is difficult to identify the epithelial infiltration into the connective tissue and the keratin pearl formation. [6]

Application of Papanicolaou stain to paraffin block tissue sections was first performed by Johnson and Klein to demonstrate keratin. Elzay and co-workers modified this technique for demonstration of keratin. Modification is achieved by increasing the staining time for three basic Papanicolaou stains and incorporating phloxine B as an additional staining component. The staining solution of modified Papanicolaou technique consisted of a nuclear stain, Harris haematoxylin. It has affinity for chromatin, attaching to sulphate groups on the D.N.A. molecule. Harris' hematoxylin is the commonest one used, although Gills' hematoxylin can also be used. Three cytoplasmic counter stains, phloxine B, orange G, and eosin azure. Phloxine B has an affinity to stain keratin. Orange G stains matured and keratinized cells. The target structures are stained

orange in different intensities. Eosin azure is composed of eosin Y, light Green SF yellowish, and bismark Brown. [7] Eosin Y gives a pink colour to cytoplasm of mature squamous cells, nucleoli, cilia and red blood cells. Staining solutions commonly used in cytology are EA 36 and EA 50, while EA 65. Light green SF stains blue to cytoplasm of metabolically active cells like parabasal squamous cells, intermediate squamous cells and columnar cells. Bismarck brown Y stains nothing and sometimes it is often omitted. [8]

The tissue stained with Papanicolaou stain showed a varied affinity to different components of the stain. The effect of orange G is evident in tissue, when keratinized cells are present. The colour of keratin range from orange to deep red depending upon the degree of keratinization, where the same distinctive nature of the stain cannot be appreciated in the slide stained with haematoxylin and eosin stain. [6]

## **MATERIALS AND METHODS**

This was a cross sectional study done in the Department of pathology and Department of MLT, Government Medical College Thiruvananthapuram. The study was done for a period of 6 months. 89 consecutive skin biopsy sections showing keratin were included in the study. The histopathology of these lesions showed a variety of neoplastic and nonneoplastic conditions like well differentiated squamous cell carcinoma, Verrucous carcinoma, verrucous hyperplasia, squamous papilloma, verruca vulgaris, Actinic keratosis, seborrheic keratosis, corn foot, epidermal and dermoidcysts. The paraffin blocks of these 89 biopsy specimens were taken and two sections of 4 microns size were cut. H &E stain and modified PAP stain was done

### **Haematoxylin and eosin staining [9]**

Deparaffinise the sections with 2 changes of xylene and Hydrate the tissue sections with descending grades of alcohol

Staining with Harris haematoxylin - 5 minute and wash with water

Differentiation with 1% acid alcohol- 1 dip and wash with water.

Blueing in running tap water-10 minute. Staining with 1% eosin--1 minute and wash with water.

Dehydrate with absolute alcohol 2 changes, Clear in xylene and Mount in DPX

**Modified Papanicolaou staining** <sup>[10]</sup>

Reagents

**Harris's hematoxylin**

- Haematoxylin 2.5 g
- Absolute alcohol 25 ml
- Potassium alum 50 g
- Distilled water 500 ml
- Mercuric oxide 1.25 g

**Phloxine - B (1% aqueous C.I no 45410)**

- Phloxine-B 1 g
- Distilled water 100 ml

**Orange G6**

- Orange G (10% aqueous) 50ml
- Alcohol 950ml
- Phosphotungstic acid 0-15g

**Eosin-azure 36**

- 0.5% Eosin y 450 ml
- 0.1% light green 450 ml
- 0.5% bismark brown 100 ml
- Phosphotungstic acid 2 g

**Staining procedure**

Deparaffinise the sections with 2 changes of xylene and Hydrate the tissue sections with descending grades of alcohol- 2 minute Stain with Harris haematoxylin- 5 minute and wash with water

Differentiation with 1% acid alcohol and wash with water.

Blueing in running tap water--10 minute.

Staining with 1% phloxine B solution-5 minute and Rinse in water

Dehydrate the tissue sections with ascending grades of alcohol

Staining with orange G6 solution- 5 minutes and Rinse in 95% alcohol, 2 changes -2 minute each

Staining with eosin azure-4 minute and Rinse in 95% alcohol, 2 changes- 2 minute each

Dehydrate with absolute alcohol 2 changes, clear in xylene and mount in DPX

Modified PAP stained sections were evaluated by comparing to H&E stain. 4 parameters were assessed. Staining quality, background clarity, morphology and differentiation. These parameters were statistically evaluated separately. Each parameter was compared using the scoring system. The scores allotted to each parameter was analysed by 'unpaired t test' at 95% confidence level (p ≤ 0.05)

TABLE 1: criteria of assessment parameters

Assessment parameters	Score given as per mentioned criteria		
	1	2	3
Staining quality	Not stained, unevenly stained, has artefacts	Details not visualised but suitable for diagnosis	Good contrast, brilliant staining
Background	Not clear, lot of deposits	Moderate deposit but suitable for study	Clear background, no interference
Morphology	Not preserved, detached from slide	Moderately preserved	Good preservation of tissue architecture
Differentiation	Specific components not seen clearly	Specific components seen well but in some area difficult to appreciate	Specific components seen clearly and appreciated very well

**RESULTS**

**a. Staining quality:**

The observed p value is 0.814 and there is no significant difference between H&E and modified PAP in aspect of staining quality.

Table 2: Comparison between staining quality of H&E and modified PAP

	H & E (N=89)		Modified PAP (N=89)		P value
	mean	SD	mean	SD	
	Staining Quality	2.6	0.52	2.58	

**b. Background clarity:**

The observed p value of background clarity in H&E stain is 0.321, there is no significant difference between H&E and modified PAP.

Table 3: Comparison of background clarity of H&E and modified PAP

	H & E (N=89)		Modified PAP (N=89)		pvalue
	mean	SD	mean	SD	
	Background clarity	2.67	0.47	2.58	

**c. Morphology:**

The observed P value of morphologic feature is 0.097 and there is no significant difference between H&E and modified PAP in aspect of morphologic feature.

**d. Differentiation:**

	H & E (N=89)		Modified PAP (N=89)		P value
	mean	SD	mean	SD	
	Morphology feature	2.51	0.5	2.46	

The comparison of differentiation feature of H&E and modified PAP shows the observed p value as 0.086. So there is no significant difference occurs between H&E and modified PAP.

**Table 5: Comparison of differentiation feature of H&E and modified PAP**

	H & E (N=89)		Modified PAP (N=89)		P value
	mean	SD	Mean	SD	
	Differentiation feature	2.48	0.5	2.38	

**DISCUSSION**

Regarding benign lesions, keratin inside the lesion of epidermal cyst show similar staining pattern to the keratin on the surface epithelium in sections stained by modified PAP. But this stained keratin inside the dermoid cyst weaker than surface keratin. In cases of corn foot, uniform staining of the keratin was not observed by modified PAP. This is similar to findings in a study by Elzay *et al*, in which uniform staining was not observed in the parakeratinized layer in modified PAP stained sections [11] The surface keratin of verrucous hyperplasia, squamous papilloma, verruca vulgaris showed good intensity of staining by modified PAP. This was similar to the study conducted by Rao *et al*. [12] other benign lesions like Actinic keratosis and seborrheic keratosis showed no significant difference in the staining quality when modified PAP was compared to H&E stain. This was in contrast to the study by S. Preethi *et al* which showed a marked difference in staining between H&E and PAP stains. [6]

In our study, modified PAP was extremely useful in the demonstration of keratin in well differentiated squamous cell carcinomas in which the differentiation or contrast was excellent when compared to H&E stain. The areas of keratinization were stained from shades of orange to pink. Study by Ramulu *et al* showed that only the central core was stained some of the keratin pearls and in some only the periphery was stained. [10] This was said to be due to defect in the maturation process of keratin. Individual cell keratinization in cases of moderately differentiated squamous cell carcinoma, were clearly demonstrated by modified PAP, which could not be appreciated much in the H&E stained sections. Cases of verrucous carcinoma showed no significant difference in staining pattern, background clarity, morphology and differentiation when the two stains are compared. This was similar to the findings of Ramulu *et al* where distinct pink was appreciated with H&E stain and magenta pink in modified PAP stained sections. [10]

**CONCLUSION**

The present study showed that the staining characteristics of modified PAP on paraffin embedded tissue sections was comparable to H and E. When the four parameters: staining pattern, background clarity, morphology and differentiation were assessed, modified PAP did not show a significant advantage over H&E. But the contrast provided by modified PAP could easily discern keratin from other connective tissue elements.

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