Research Article

Immunocytochemical staining of cervical smears – A comparative study with routine cytology for confirmation of precancerous and cancerous lesions of cervix

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Abstract

Introduction: Carcinoma cervix is one of the leading causes of morbidity and mortality. The most effective method of screening is based on Pap smear study. However, Pap smear has relatively low sensitivity and irreducible false negative & false positive rates. In countries like India, it is not always feasible to follow the patient for confirmation by histology. In the present study, we have tried to fill this gap by staining the cervical smears with, epithelial cell markers including CK17 and CEA expressed by dysplastic squamous cells. **Materials & Method**s: 50 cervical smears reported as precancerous or cancerous were stained with anti CK17 and anti CEA. The patients were followed for histopathology. **Results:** Out of 20 cases of ASCUS, three were positive both for anti-CK17 and anti-CEA and six were positive for anti-CK17 only. Out of 18 cases of LSIL, nine were positive for both anti-CK17 and anti-CEA and fifteen were positive for anti-CK17 only. Out of eight cases of HSIL, three were positive for both anti-CK17 and anti-CEA and six were positive for anti-CK17 only. Out of 4 cases of SCC, three were positive for both anti-CK17 and anti-CEA and all four were positive for anti-CK17. **Conclusion:** Positive result in staining was higher in high grade lesions. CK 17 was found to be better marker than CEA. CK17 and CEA can be used as adjunct to Pap smear screening but its high cost may restrict its use in routine.

Key words: Cervical Smears, Immunocytochemistry, CK 17, CEA

Introduction

Worldwide, carcinoma of the uterine cervix is the second common type of malignancy in women after carcinoma breast. Approximately 4,71,000 new cases of the cervical carcinoma are detected each year throughout the world. About 80% of cases are found in developing countries. Cervical carcinoma kills about three lacs women each year although it is completely preventable disease [1-3].

There is a declining trend in incidence and mortality rates of cervical carcinoma in developed countries over the past four decades which is mainly attributable to the implementation of organized screening programs based on the Pap smear test which was introduced in 1941 at Cornell University United States [4-7]. The Pap smear test has reduced the overall death rate by approximately 74% and annual deaths by approximately 2%. Deaths due to carcinoma cervix are still higher in populations around the world where women do not have routine screening by Pap smears tests. [8-11].

The Pap smear test, however, is limited with respect to its sensitivity and specificity [6]. The false-negative rate for cervical premalignant lesions and cervical carcinoma lies between 15–50% and false-positive rates of approximately 30% have been reported to date [12]. This limits present screening programmes and emphasizes the need for identification of specific

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biomarkers for dysplastic epithelial cells to aid in primary screening and lesion diagnosis.

During the past several decades there are remarkable advances in immunological staining of cells i.e. immunocytochemistry by employing antibodies as specific probes for the visualization of cell and tissue bound antigens have literally revolutionized the practice of pathology.

The present work is undertaken to study immunocytochemical pattern of cervical smears and to compare the result of staining with routine cytology. This also includes studying the extent to which immunocytochemical staining helps in morphological analysis to reach at correct diagnosis of various precancerous lesions of cervix.

Aims and objectives

1) To study the pattern of precancerous and cancerous lesions of cervix in patients attending C. R. Gardi Hospital Ujjain.
2) To study and compare the routine cytology and immunocytochemistry in precancerous and cancerous lesions of cervix.
3) To study the efficacy of immunocytochemistry in diagnosis of various lesions of cervix.

Methods and Materials

The study was conducted in the Department of Pathology, Ruxmaniben Deepchand Gardi Medical College, Ujjain between August 2011 to September 2012.

Cervical smears from patients attending out-patient and in-patient department of Obstetrics and Gynecology of Chadrikaben Rashmikant Gardi hospital were received in cytology laboratory. The cervical smears were stained with Papanicolaou stain and reported according to “The Bethesda System” 2001 [13].

Cervical smears with precancerous and cancerous lesions of cervix were enrolled in the study. Two additional cervical smears were collected from the enrolled patient after their consent on Poly-L-Lysine coated slides and were fixed in methanol. The slides were stored in refrigerator at 2-8°C until stained.

The patients were followed for histopathology. Biopsy or hysterectomy specimen were obtained and processed for cyto & histopathology correlation.

Cervical smears reported as inflammatory smears, cervical smears obscured by mucus or blood were excluded before enrollment and patients without histopathology reports were excluded from the study.

ICC was performed using Polymeric (Envision TM Flex mini kit DAKO 8023) technique on cervical smear preparation. Both the antibodies used in the study were optimally pre-diluted and were ready to use. The staining kit was provided by Dako (Code number 8023) (Table I).

Table-I: Antibodies used in the study

Antibody	Source	Clone	Chromogen
Anti-carcinoembryonic antigen	Dako (IS622)	Monoclonal (Mouse)	Diaminobenzidine
Anti-cytokeratin 17	Dako (IS620)	Monoclonal (Mouse)	Diaminobenzidine

Quality control- Each staining run included a known positive control smear and negative control smear.

Reading of results-Smears with more than 5 morphologically intact cells showing cytoplasmic staining (brown) are considered positive for both cytokeratin17 and carcinoembryogenic antigen expression.

Result

On cytology, 20/50 (76%) cases were of ASCUS, 18/50 were of LSIL, while HSIL were observed in remaining 08/50 (16%) cases and SCC in 4/50 (08%) cases (Table- II).

Table –II: Pattern of precancerous and cancerous lesions of cervix on cytology (N=50)

Cytology lesions	Number (%)
ASCUS*	20 (40)
LSIL†	18 (36)
HSIL‡	08 (16)
SCC§	04 (08)
Total	**50 (100)**

*Atypical squamous cell of undetermined significance
†Low- grade squamous intraepithelial lesion
‡High-grade squamous intraepithelial lesion
§Squamous cell carcinoma

ASCUS is almost evenly distributed across age ranges below 50 years, while 11/18 (61.1%) LSIL cases were in the age range of 31-40 years. In HSIL and SCC majority of cases were in the higher age range of 41-50 years.

Among all the 32/50 (64%) females who had first coitus before 20 years of age, 25/32 (78.1%) cases had ASCUS or LSIL while 16/50 (32%) cases who had first coitus in the age range of 20-29 years, 12/32 (75%) cases had either ASCUS or LSIL. 4% were not aware of their age at first coitus.

Thirty seven (74%) patients were multiparous, while remaining 13/50 (26%) were multiparous with history of abortion. In the former group majority i.e. 29/37 (78.4%) cases had ASCUS or LSIL (Table- III).

Table-III: Demographic characteristics of cases enrolled in the study (N=50)

Demographic variables	Cytology lesions				
	ASCUS*	LSIL†	HSIL‡	SCC§	Number (%)
Age (in years)					
20-30	05	03	00	00	08 (16)
31-40	06	11	02	01	20 (40)
41-50	07	02	04	03	16 (32)
≥51	02	02	02	00	06 (12)
Religion					
Hindu	17	17	07	04	45 (90)
Muslim	03	01	01	00	05 (10)
Age at coitus (in years)					
09-19	12	13	05	02	32 (64)
20-29	07	05	02	02	16 (32)
Unknown	01	00	01	00	02 (04)
Obstetrics history					
Multiparous	17	12	06	02	37 (74)
Multiparous with H/O of abortion	03	06	02	02	13 (26)

*Atypical squamous cell of undetermined significance
†Low- grade squamous intraepithelial lesion
‡High-grade squamous intraepithelial lesion
§Squamous cell carcinoma

Histology on cervical biopsy or hysterectomy specimen showed normal histology in 14/20 (70%) cases of ASCUS. Out of 18 LSIL cases, two (11.11%) cases showed normal histology, while 8/18 (44%) showed CIN1. Out of eight HSIL

cases, three (37.5%) showed CIN3, while five (62.5%) cases showed SCC. The cytology of SCC showed similar finding on histology (Table - IV).

Table –IV: Histology finding in precancerous and cancerous lesions of cervix

Histology findings	**Cytology lesions**				
	ASCUS*	**LSIL†**	**HSIL‡**	**SCC§**	**Number (%)**
Normal histology	14	02	00	00	16 (32)
CIN¶1	05	08	00	00	13 (26)
CIN 2	01	04	00	00	05 (10)
CIN 3	00	04	03	00	07 (14)
SCC	00	00	05	04	09 (18)
Total	20	18	08	04	50 (100)

*Atypical squamous cell of undetermined significance
†Low- grade squamous intraepithelial lesion
‡High-grade squamous intraepithelial lesion
§Squamous cell carcinoma
¶Cervical intraepithelial neoplasia

Out of 20 cases of ASCUS, three (15%) were positive both for anti-CK17 and anti-CEA and six (30%) cases were positive for anti-CK17 only. Out of 18 cases of LSIL, nine (50%) were positive for both anti-CK17 and anti-CEA and 15 (83%) were positive for anti-CK17 only. Out of eight cases of HSIL, three (37.5%) were positive for both anti-CK17 and anti-CEA and six (75%) were positive for anti-CK17 only. Out of 4 cases of SCC, three (75%) were positive for both anti-CK17 and anti-CEA and all four (100%) was positive for anti-CK17.

It is evident that as the grade of cytology lesions is increasing, positive result for both the markers is also increasing (Table V).

Table- V: Positive results of anti- CK17 and anti-CEA in precancerous & cancerous lesions of cervix

Cytology category	**Anti-CK 17 n (%)**	**Anti-CEA n (%)**	**Both\ n (%)**
ASCUS *(n=20)	06 (30)	03 (15)	03 (15)
LSIL† (n=18)	15 (83)	09 (50)	09 (50)
HSIL‡ (n=8)	06 (75)	04 (50)	03(37.5)
SCC§ (n=4)	04 (100)	03 (75)	03(75)

*Atypical squamous cell of undetermined significance
†Low- grade squamous intraepithelial lesion
‡High-grade squamous intraepithelial lesion
§Squamous cell carcinoma

To determine the efficacy of CK17 and CEA expression, a comparison of result of immunocytochemical staining was carried out with the histology being considered as gold standard. (Table VI, VII).

31/34 (91.2%) smears were found positive and 03/34 (8.8%) were found to be negative on immunocytochemical staining with anti-CK17. This resulted in sensitivity of 91% and specificity of 56%. The positive predicted value (PPV) was 81.5% and the negative predicted value (NPV) was 75%. The statistical significance of association between the CK 17 expression and histology findings was tested using Fisher exact test, which indicated significant association between the two (p value=0.0006).

Table-VI: Association between anti-CK17 and histology finding

Anti-CK 17	Histology finding		Total
	Positive	**Negative**	
Positive	31	07	38
Negative	03	09	12
Total	**34**	**16**	**50**

Similarly, 19/34 (55.8%) were found to be positive and 15/34 (44%) were found to be negative on immunocytochemical staining with anti-CEA. The sensitivity was obtained as 56% while specificity was 100%. The positive predicted value (PPV) was 100%, while the negative predicted value (NPV) was 51%.

There was statistical significant association between CEA expression and histology when it was evaluated by using Fisher's exact test (p-value < 0.0001).

Table-VII Association between anti-CEA and histology findings

Anti-CEA	Histology finding		Total
	Positive	**Negative**	
Positive	19	0	19
Negative	15	16	31
Total	**34**	**16**	**50**

Comparison of CK17 and CEA expression (Table VIII)

For being the diagnostic marker for precancerous and cancerous lesions of cervix, its sensitivity is expected to be high. Expression of CK17 had higher sensitivity (91%) as compared to that of CEA expression (56%). Also, the NPV for CK17 expression was higher (75%) as compared to CEA expression (51%), thereby CK17 expression giving a better reassurance that smear does not have a precancerous and cancerous lesion.

Table – VIII: Association of histology with different markers

ICC Markers	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Anti-CK17	91	56	81.5	75
Anti-CEA	56	100	100	51
Both	100	100	100	100

All statistical parameters were higher when both the markers were used in combination rather than used alone.

Discussion

Cervical cancer is the most common cancer in women in developing countries [14]. 86% of cancer deaths occur due to cervical cancer in developing, low- and middle-income countries [15-17].

Every year in India, 1,22,844 women are diagnosed with cervical cancer and 67,477 die from the disease. India has a population of 432.2 million women aged 15 years and older who are at risk of developing cancer. India also has the highest age standardized incidence of cervical cancer in South Asia at 22, compared to 19.2 in Bangladesh, 13 in Sri Lanka, and 2.8 in Iran [18].

One of the most effective ways of preventing and controlling cervical carcinoma is regular screening and early diagnosis. The most effective method of screening is based on Pap smear study, which has contributed considerably in reducing the incidence and mortality [6].

However, Pap smear has relatively low sensitivity and irreducible false negative & false positive rates [6, 12]. Pap smear is based on cytology and is presumptive test. So, it has to be confirmed by histology examination. In countries like India, it is not always feasible to follow the patient for confirmation by histology. Moreover,

histology itself being an invasive test creates a gap between screening and confirmation.

In the present study, we have tried to fill this gap by using epithelial cell markers including CK17 and CEA expressed by dysplastic squamous cells by staining the smear with these markers. We investigated the extent to which these makers can act as adjunct to screening with Pap smear.

In our study 50 cases of precancerous and cancerous lesions of cervix were evaluated. We found majority of cases, 20/50 (40%) were ASCUS. Study by GPS Yeoh et.al. showed 2.29%, Mandakini et.al. showed 04% and Mihaela et.al. showed 48% cases of ASCUS [19, 20, 21]. No cases of AGUS were found in our study and in study by GPS Yeoh et.al. and by Mihaela et.al [20, 21]. Study by Mandakini et.al. showed 5% cases of AGUS [19]. No cases of ASC-H were found in our study and in study by GPS Yeoh et al [20]. Study by Mandakini et. al. showed 5% and Mihaela et. al. showed 07% cases of ASC-H [19, 21]. Out of 50, 18 (36%) cases were LSIL in our study. Study by GPS Yeoh et. al. showed 65%, Mandakini et. al. showed 10% and Mihaela et.al. showed 23.6% cases of LSIL [19, 20, 21]. Eight out of fifty (16%) cases were HSIL in our study. Study by GPS Yeoh et.al. showed 46%, Mandakini et.al. showed 1% and Mihaela et.al. showed 06% cases of HSIL [19, 20, 21]. Four (08%) cases were of SCC in our study. Study by GPS Yeoh et.al. showed 08%, Mandakini et.al. showed 7% cases of SCC [19, 20]. No cases of SCC were found in study Mihaela et.al [21].

As compared to the study by GPS Yeoh et al, Mandakini et.al. and Mihaela et.al incidence of various cytology lesions was found comparatively high in our study because we had excluded the cases reported as NILM or unsatisfactory on cytology [19, 20, 21]. When the cases reported as NILM or unsatisfactory were excluded from the study by GPS Yeoh et.al. , Mandakini et.al. and Mihaela et.al. Incidence of these cases in various cytology categories found to be comparable with other studies [19, 20, 21].

However, the numbers of cases of ASCUS in our study were near to that of study done by Mihaela et.al [21]. Similarly numbers of cases of SCC were equal in our study and in study by GPS Yeoh et.al [20].

In our study ASCUS, HSIL and SCC were highest in age group of 41-50 years and LSIL was highest in age group of 31-40 years. In study by Mandakini et.al. ASCUS and LSIL was highest in age group of 41-50 years and AGUS, HSIL and SCC were highest in age group of 31-40 years [19]. No age wise distribution was found in study by GPS Yeoh et.al. and Mihaela et.al [20, 21]. Study by Mandakini et.al. showed high grade lesions in reproductive age group as compared to our study showing high grade lesions in premenopausal and menopausal age group [19]. However the cases of ASCUS were also high in older age group, suggesting the frequent screening and follow up of older patients in our set up.

In our study 16/50 (32%) cases were false positive. 14/16 (87.5%) cases were reported as ASCUS and 02/16 (12.5%) as LSIL on cytology. In various studies there is wide variation in false positive rates from 5-40% [19, 20, 21].

Out of 20 cases of ASCUS, 70% showed normal histology, 05 (20%) showed CIN 1 and 01/20 (5%) showed CIN2. Two out of 18 cases, 11.11% cases of LSIL were false positive being negative for any abnormal histology, 08/18 (44%) showed CIN 1, 04/18 (22%) showed CIN 2 and 04/18 (22%) showed CIN 3. Out of eight cases, three (37.5%) cases of HSIL showed CIN 3 and 05/08 (62.5%) cases showed SCC. Thus cases of HSIL either showed either showed high grade lesion or frank malignancy. All four (100%) cases showed SCC on histology. So the true positive result in SCC was highest and 100% and the false positive results were highest in ASCUS. This may be due to the presence of inflammatory or degenerative changes, air drying artifact with nuclear enlargement. In case of difficulty patient's age, history should be considered. Previous specimen should be reviewed. As grading of lesions was increasing false positive results decreased and were almost nil for HSIL & SCC.

In study by Yeoh et.al. showed, out of total cases of ASCUS, 9% showed normal histology, 45% showed mild atypia, 28% showed CIN 1, 15% showed either CIN 2 or CIN 3and 1.5% were SCC [20]. Out of total cases of LSIL, 3% showed normal histology, 1% showed mild atypia, 45% showed CIN 1, 39.5% showed either CIN 2 or CIN 3 and 1% showed SCC [20]. Out of total cases of HSIL, 7% showed normal histology, 1% showed mild atypia, 18% showed CIN 1, 69.2% showed either CIN 2 or CIN 3and 3% showed SCC. Out of total cases of SCC, 40% showed either CIN 2 or CIN 3and 60% showed SCC [20].

In study by Mihaela et.al. showed out of total cases of ASCUS, 61% showed normal histology, 23% showed CIN 1, 13% showed CIN 2and 3% showed CIN 3. Out of total cases of LSIL, 11% showed normal histology, 61% showed CIN 1, 26% showed CIN 2, 1% showed CIN 3 [21]. Out of total cases of HSIL, 26% showed CIN 1, 21% showed CIN 2, 53% showed CIN 3[21].

No cyto-histology correlation was done in study by Mandakini et.al [19]. As compared to study by Yeoh et al and Mihaela et.al. it was clear that as grade of lesion on cytology increasing, the false positive result decreasing as that seen in our study [20, 21].

False negative results could not be obtained as our study excluded the smears reported as NILM. Study by Yeoh et.al. showed 7.81%, Sodhani et.al. 0%, Saha et.al. showed 27.45% of false negative results [20, 22, 23].

On immunocytochemical staining with anti-CK17, out of 34 cases with abnormal histology finding, 31/34 (91.2%) were found to be CK 17 positive and 03/34 (8.8%) were found to be negative. It was found that as the grade of lesion increases on histology, the intensity and number of cells with stain were also higher on cytology. All cases of ASCUS and SCC with abnormal histology were positive for anti-CK17 whereas 6/8 (75%) & 14/18 (77.8%) cases of HSIL & LSIL with abnormal histology were positive for anti-CK17 respectively. So at the extremes of cytology lesion positive results for anti-CK17 was 100%.

On the other hand 16 cases with normal histology, 07/16 (43.7%) were positive for anti-CK17. This may be due to the presence of inflammatory or degenerative changes, air drying artifact with nuclear enlargement on cytology resulting in reporting of cases as ASCUS or LSIL or due to non representative cervical biopsy or due to drying artifacts during immunocytochemistry. Nine out of sixteen (56.25%) were negative for CK17 expression.

Further, the distribution resulted into sensitivity of 91% (95% CI: 0.75 – 0.97) and specificity of 56% (95% CI: 0.30 – 0.79). The high sensitivity of CK17 expression was because of small false negatives (3). In other words, the CK17 expression shows high probability of detecting positive cases correctly. The lower specificity of the CK17 expression was because of higher number of false positive cases (7) compared to true negatives (9). In other words, CK17's probability of detecting negative cases correctly is low because of higher false positives. The positive predicted value (PPV) of the anti- CK17 was 81.5%. It tells how likely the cases identified as positive by anti-CK17 are diseased (as per gold standard). Further, the negative predicted value (NPV) of the marker was 75%, which tells how likely the cases identified as negative by marker do not have disease (as per gold standard).

In study by Frank Smedts et.al. showed that out of total cases, small percentage of CIN 1 and CIN 2 lesions showed CK17 but 51% of CIN 3 showed positive results for CK 17 [24]. The scientists proposed that CIN 3 lesions expressing CK 17 may be of the progressive type and CK 17 could indicate whether CIN 3 lesion will become malignant or not [24]. In the same study it was found that CK 17 was positive in 100% cases of adenocarcinoma and SCC as in our study [24]. However we did not find any case of adenocarcinoma of cervix.
As in our study as grade of lesion on histology was increased, the positive results for CK17 expression were increased being 100% for SCC which is same as that of study by Frank Smedts et.al [24].

On immunocytochemical staining with anti-CEA, out of 34 cases with abnormal histology finding, 19/34 (55.8%) were found to be positive and 15/34 (44%) were found to be negative. On the other hand 16 cases with normal histology, 16/16 (100%) were negative.

The sensitivity of CEA expression was obtained as 56% (95% CI: 0.38 – 0.72), while specificity was 100% (95% CI: 0.75 – 1.00). The low sensitivity was due to more false negatives (15). The specificity was 100 % as there were no false positives identified by anti-CEA. The positive predicted value (PPV) was 100%, while the negative predicted value (NPV) was 51% (95% CI: 0.33 – 0.69). The lower NPV was due to higher false negatives as yielded by anti-CEA.

In study by Aron Tendler et.al., anti-CEA staining intensity was significantly increased in high-grade squamous lesions (CIN III and SCC) compared with normal cervical mucosa and CIN grades I or II. There was a linear correlation between grade of lesion and staining intensity. CEA expression increased most significantly between CIN grades 2 to 3. Only 1 of 7 primary cervical adenocarcinoma expressed CEA. CEA expression may be a useful diagnostic tool for those at risk for progressive cervical neoplasia [25].

Toki and Yajima et.al. investigated the relationship of the positive pattern of anti-CEA staining in squamous cell carcinomas of the cervix, to the prognosis of the patients with the neoplasm. Four patterns of localization of CEA were observed depending on the location of CEA - positive areas in cancer nests: central type, surrounding type, diffuse type and focal type. The prognosis of patients was most excellent in the disease of central type, diffuse type and focal type [26].

To sum up, studies done on expression of CEA and CK17 shows definite positive result in carcinoma and mixed results with low grade lesions as we found in our study.

Conclusion

Majority of cervical smears were found to be of low grade lesions. As the grade of lesion increases on cytology, number of cases was found to be decreased. High grade lesions show somewhat more incidence in higher age group as compared to low grade lesions. So, the screening programmes should be targeted in older age group. Cyto-histology correlation was to be found to be higher in high grade lesions as compared to low grade lesions. Positive results of staining with anti-CK17 & anti-CEA was higher in cancerous lesions rather than precancerous lesions. Among the two markers CK17 was found to be better marker than CEA. The results were enhanced with both anti-CK17 and anti-CEA were used in conjunction rather used alone. CK17 and CEA in combination can be used as adjunct to Pap smear screening but its high cost may restrict its use in routine.

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