Original Article

Utility of immunohistochemistry in demonstrating *Helicobacter pylori*

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Abstract

Background: *Helicobacter pylori* is the causative organism for chronic active gastritis, duodenal ulcer and also for malignancies like gastric adenocarcinoma and mucosa associated lymphoid tissue lymphoma. It is essential to demonstrate the presence of *H. pylori* in gastric biopsies as it has an important role in patient care. Though there are several special stains to detect *H. pylori* in histological sections, the specificity and sensitivity vary greatly. Immunohistochemically *H. pylori* can be detected by using anti *H. pylori* antibody, which reacts with somatic antigens of the whole bacteria. The aim of this study was to compare the reliability of routine hematoxylin and eosin (H and E), Giemsa, Warthin–Starry (WS) silver stain and immunohistochemical technique in diagnosing *H. pylori*. **Materials and Methods:** In this retrospective 5-year (2008) study, endoscopic gastric biopsies taken from patients during gastroesophageal endoscopy with histopathological diagnosis of gastritis were studied. Standard H and E staining was performed on 5-μm-sections from paraffin block of each specimen. Microscopic sections of biopsy specimens of patients showing features of gastritis histopathologically in routine H and E stain and where the presence of *H. pylori* was suspected were also stained with Giemsa, WS, and immunohistochemistry (IHC) using purified polyclonal *H. pylori* antiserum (Biocare). We included gastric resection specimens in our study. **Results:** Of the 25 cases, 25 (100%) showed presence of *H. pylori* on H and E, Giemsa and WS stains, whereas 24 (96%) cases demonstrated *H. pylori* on IHC stain. **Conclusion:** We conclude that *H. pylori* detection by IHC has advantage over routine H and E staining. However, in the developing countries with financial constraints, routine H and E staining in combination with special staining are fairly reliable in demonstrating *H. pylori*.

Key words: Giemsa stain, *Helicobacter pylori*, hematoxylin and eosin stain, immunohistochemistry, Warthin–Starry stain

INTRODUCTION

Helicobacter pylori is the causative organism for chronic active gastritis, duodenal ulcer and malignancies like gastric adenocarcinoma and mucosa associated lymphoid tissue (MALT) lymphoma.[1,2] It resides in the mucus layer of the gastric mucosa.[3,4] It is essential to demonstrate the presence of *H. pylori* in gastric biopsies as it has an important role in patient care. Due to the therapeutic implications, over the years pathologists have sought more reliable methods for detecting *H. pylori* in biopsy specimens, including immunohistochemistry (IHC), polymerase chain reaction (PCR) and more recently, *in situ* hybridization.[5] Histopathological assessment of the antral biopsy specimens is an easy and cost-effective method for diagnosing *H. pylori* infection. Though there are several special stains to detect *H. pylori* in histological sections, their specificity and sensitivity vary greatly.[6] Notable among these special stains are silver impregnation stains and modified Giemsa (MG) stains.[5,6]

Immunohistochemically *H. pylori* can be detected by using anti *H. pylori* antibody which reacts with somatic antigens of the whole bacteria.[4] The aim of this study was to compare the reliability of routine hematoxylin and eosin (H and E) stain with Giemsa, Warthin–Starry (WS) silver stain and immunohistochemical technique in diagnosing *H. pylori*.

MATERIALS AND METHODS

In this retrospective 5 year (2008) study, endoscopic gastric biopsies taken from patients during gastroesophageal endoscopy with histopathological diagnosis of gastritis were studied.

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The specimens were fixed in 10% formal saline for 24 h and then dehydrated in increasing concentrations of isopropyl alcohol followed by, clearing of alcohol by xylene before impregnating in paraffin wax. The specimens were subsequently embedded in paraffin wax in cassettes to facilitate tissue sectioning. Standard H and E staining was performed on 5-μm-sections from each specimen block. Histological sections of biopsy specimens of patients (formalin-fixed and paraffin-embedded), with histopathological evidence of gastritis or with the suspicion of presence of *H. pylori*, were also stained with the Giemsa, WS, and IHC using purified polyclonal *H. pylori* antiserum (BioGenex). Each biopsy section was carefully examined for the presence of *H. pylori*. The data obtained was given in the form of simple percentages. Statistics was done by using Chi-square method. We have not included gastric resection specimens in our study.

RESULTS

There were 79 cases of histopathologically diagnosed gastritis in the study period. There was slight male predominance in our study group with M: F ratio of 1.2:1. The mean age was 47.2 years. The gastric biopsies were classified according to Sydney classification[7] [Table 1]. *H. pylori* was detected in 49 (62%) cases. Routine H and E and special stains like Giemsa and WS detected *H. pylori* in 26 (32.9%) cases [Figures 1-3]. Statistical analysis done by Chi-square test showed both special stains and immunostains to be comparable and independently good. Immunostaining detected additional 29 cases which were not detected initially by routine H and E or special stains [Figure 4]. Here, however very few bacilli were detected by immunohistochemical method. Immunostains were negative in six cases where *H. pylori* was suspected by routine methods. The sensitivity and specificity for special stains was 100% and 90% respectively. IHC showed 100% sensitivity and 51% specificity. Positive predictive value for special stains and IHC was 77% and 41%, whereas negative predictive value for both was 100% [Table 2].

DISCUSSION

Helicobacter pylori infection is common in the Indian subcontinent. Exposure occurs in childhood and approximately 80% of Indian adults have been infected at some point in time.[8] In a study from South India, the authors have concluded that *H. pylori* infection is very common in the South Indian population. A high prevalence is seen in all gastroduodenal diseases and more than half the population without any abdominal symptoms was colonized by the *H. pylori*.[9]

In this study, *H. pylori* was detected in 62% of gastritis cases.

Helicobacter pylori is a Gram-negative, spiral organism, which colonizes the gastric mucosa.[10] *H. pylori* infection is associated with gastritis, gastric ulcer, gastric adenocarcinoma, and MALT lymphoma.[1,2] Therefore, it is useful to document the presence of *H. pylori* in a gastric biopsy for giving appropriate patient care. *H. pylori* survives in the acidic medium of stomach by a number of mechanisms. It secretes the urease enzyme, which converts urea to ammonia. The production of ammonia around *H. pylori* neutralizes the acidity of the stomach, making the

Table 1: Histological findings and *H. pylori* detection

Histology	Number of case (n=79)	*H. pylori* detection (n=49)
Mild antral superficial gastritis	4	2
Moderate antral superficial gastritis	55	38
Mild antral pan gastritis	1	1
Moderate antral pan gastritis	3	1
Severe antral pan gastritis	16	7

H. pylori: *Helicobacter pylori*

Table 2: Study results and statistics

Stain	Sensitivity	Specificity	PPV	NPV
H and E, MG, WS	100	90	77	100
IHC	100	51	41	100

H and E: Hematoxylin and eosin, MG: Modified Giemsa, WS: Warthin-Starry, IHC: Immunohistochemical stain, PPV: Positive predictive value, NPV: Negative predictive value

Figure 1: Hematoxylin and eosin stained section showing suspicious organisms over the mucosal layer (H and E, ×400)

Figure 2: Modified Giemsa stain showing few bacilli (modified Giemsa, ×400)

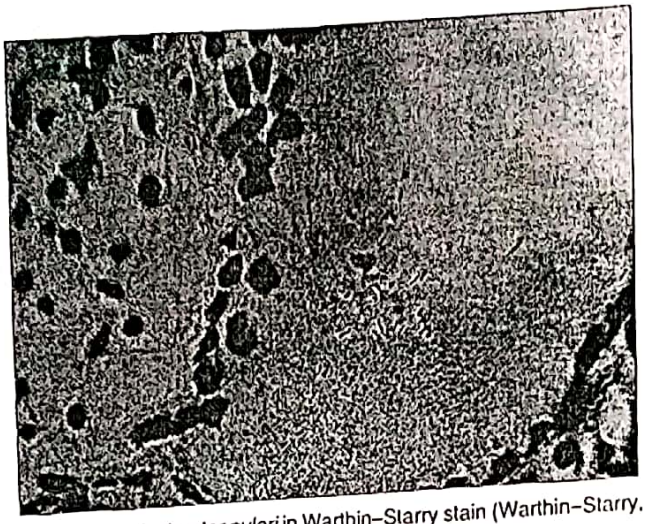

Figure 3: *Helicobacter pylori* in Warthin–Starry stain (Warthin–Starry, ×400)

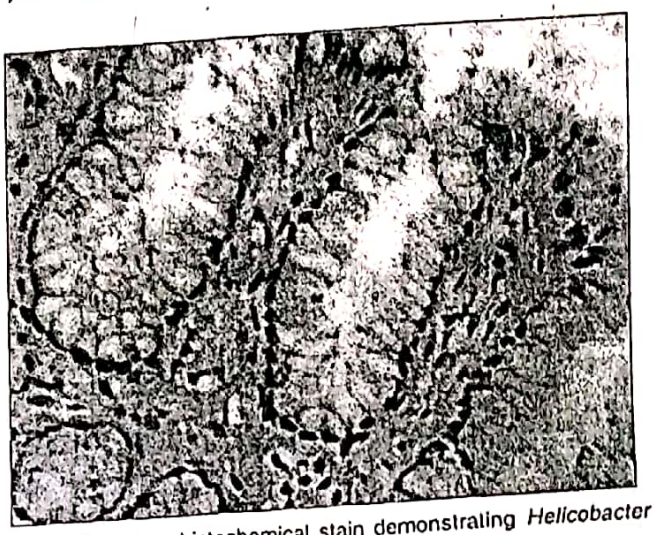

Figure 4: Immunohistochemical stain demonstrating *Helicobacter pylori* (immunohistochemistry, ×400)

medium alkaline, this being more suitable for its survival. In addition, because of its helical shape the *H. pylori* can burrow into the mucus layer, which is less acidic than the lumen of the stomach. Besides these, *H. pylori* have also developed means of interfering with local immune responses, making them ineffective in eliminating the bacteria.[11,12]

Several methods have been described for the detection of *H. pylori* including both noninvasive and invasive diagnostic tests such as serology, culture, rapid urease test,[13] C-urea breath test, and histology based on endoscopy. Though many of these tests have advantages over histopathology as being noninvasive, more rapid, and less expensive, still histological detection of *H. pylori* in a gastric biopsy remains the most common and the most sensitive test.[14,15]

Helicobacter pylori infection is widely diagnosed by means of histopathological examination. For this, apart from the routine H and E, several special staining methods like MG, WS, Gimenez, Genta, and immunohistochemical *H. pylori* antibody stains are used. Only few studies have investigated the sensitivity and specificity of the different staining methods.[5,6,14]

However, several studies found that none of these stains is specific for *H. pylori* and they may be difficult to interpret because of the nonspecific staining of mucus, debris and water bath contaminants. The WS stain, which is considered to be the most sensitive, is technically demanding and is often not reproducible. Optimal interpretation of these stains requires careful examination of the sections at high magnification. More recently, IHC, *in situ* hybridization and the PCR have been proposed as alternative specific detection methods.[15] IHC is considered as the "gold standard" for histology, being a highly sensitive and specific staining method.[5]

The commonly used H and E slide review had a very good sensitivity and specificity with all levels of observers.[16] Its advantages include its adequacy for the initial assessment of gastric biopsies in symptomatic upper gastrointestinal patients. This is because it is a well-tested, inexpensive and easy staining method, requiring a relatively short period of time to perform, with highly reproducible results. In addition, assessment of morphological changes accompanying *H. pylori* infection can be done simultaneously.[14] However, some authors are of the opinion that test performance characteristics of H and E stain are inferior to Giemsa, Genta, or silver stains.[17] According to Rotimi *et al.*, the MG stain is the method of choice because it is sensitive, cheap, easy to perform, and reproducible.[13] Sometimes, however, these bacteria were masked in both H and E and Giemsa stained sections by inspissated mucus or by being positioned flat, in close approximation to the epithelial surface. But in immunostained preparations, the organisms including coccoid forms, become more prominent.[13] In their study Rotimi *et al* they have found that by using heating method for antigen retrieval rather than trypsin, the problem of excessive background staining of epithelium and mucus, seen in IHC stain can be overcome. According to them, immunoperoxidase method is easy to use, less demanding than WS staining, and that it produces reliable results, which are easy to interpret. Low numbers or even single organisms, often difficult to detect using traditional stains, are easily identified in immunostained sections.[11,13]

In our study, we have used heating method for antigen retrieval Immunostains detected a total of 49 cases, including additional 29 cases, which were not detected initially by routine H and E or special stains. IHC was positive even when the number of bacilli was very low. This may be the reason for *H. pylori* not being initially detected by means of routine H and E stain in these specimens. On the other hand, immunostains were negative in six cases where *H. pylori* was detected by routine methods.

Recently, the detection of *H. pylori* is declining as a result of use and misuse of antibiotics. As a result, often pathologists face a scenario, where they are expected to find *H. pylori* based on history and histological findings, but the organism remains elusive in routine as well as in special stains. In these cases, IHC may play a role in detecting the organism.[18]

The dilemma faced by the pathologists is well-analyzed by the article "*H. pylori* – To Stain or Not to Stain?" by Smith *et al*.[19]

concluded that identifying the organism on immunostain was much easier and less time-consuming. However, this may not be feasible in the presence of financial constraints. Another aspect is that, in their study, there was no significant difference between the resident and faculty member in identifying the organisms stained by IHC method.[19] By using immunostains, the interobserver variation, as reported by few other studies also can be reduced.[20]

Another study by Hartman and Owens has compared the routine stains and IHC.[21] The authors have noted that the sensitivity of special stains in their study was 62% and that of IHC was 97-100%. In our study, the sensitivity of both special stains and IHC was 100%. The specificity for special stains in their study was around 97-98% and for IHC it was 100%. We had observed 90% specificity for special stains and 51% specificity for IHC.[21]

One more study, using culture as a standard, has reported sensitivity for *H. pylori* as 90.0 ± 10.0% with MG, 70.0 ± 14.1% with WS, and 83.8 ± 11.1% with IHC using purified polyclonal *H. pylori* antiserum (DAKO B471). Specificity reported was 53.8 ± 19.3%, 82.5 ± 9.6% and 90.0 ± 0.0%, respectively.[22] In our study, we have taken IHC as standard and culture was not done in the cases included in the study.

Pathologists also should be aware of other causes of gastritis that may mimic *H. pylori* infection, which include reactive gastropathy with focal activity, focally active gastritis and carditis, autoimmune gastritis, granulomatous gastritis, lymphocytic gastritis, and other infections.[18] In these conditions, the stains for *H. pylori* help to rule out other similar conditions.

In our study, the clinical presentation was variable and in many cases the clinicians requested for *H. pylori* identification. We have not included data about previous treatment received by the patients, while evaluating these staining methods. A further study taking into account various clinical parameters and the association of *H. pylori* will be definitely helpful.

We conclude that routine H and E with the help of special stains reliably help in the detection of *H. pylori*. However, *H. pylori*, by means of IHC is more easily detected compared with the conventional methods. We agree with Wang *et al.*, that IHC should be used judiciously for example in case of unexplained gastritis or in previously treated patients with low dose of organism, particularly in developing countries.[16]

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Multiple primary cancers: An enigma

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Abstract

Background: Incidence of multiple primary cancers though uncommon, is being frequently reported now-a-days owing to better diagnostic techniques as well as prolonged life span and the increased incidence of long-term survival of cancer patients. **Materials and Methods:** This is a retrospective study. Cases of multiple malignancies diagnosed histopathologically were retrieved from the archives of department of surgical oncology. Clinical data were obtained from the medical records. They were categorized as synchronous malignancies if the interval between them was less or equal to 6 months and metachronous, if the interval was more than 6 months. **Results:** A total of 13 cases were encountered in the 5 year study period. Out of them, two were in the metachronous category and the rest were synchronous as the 2nd malignancy was detected mostly during clinical evaluation of the patients for the primary malignancy. There was female predominance with age range being 43-68 years. Majority of the cases were in the 7th decade. The most common organ involved was breast, followed by cervix. Apart from bilateral breast malignancies, there were combinations like breast with cervix, endometrial carcinoma, cervical carcinoma and even papillary thyroid carcinoma. **Conclusion:** Detection of multiple primary malignancies is becoming increasingly common in day-to-day practice. Greater awareness of this is required among both cancer patients and their treating clinicians.

Key words: Dual malignancies, metachronous malignancy, multiple primary cancers, synchronous malignancy

Introduction

Multiple primary cancers are usually defined as primary malignant tumors of different histological origins in one person. Recently, there has been an increase in the number of patients diagnosed with multiple primary cancers. This trend can be attributed to improved diagnostic techniques, prolonged life span and the increased incidence of long-term survival of patients with malignancy. Most multiple primary cancers are double primary cancers.[1,2]

Definitions and classifications for multiple primary cancers and multi-centric cancers, proposed by Moertel way back in 1977 hold true even today.[2,3] Accordingly, group I includes, multiple primary cancers occurring in organs with the same histology, group II includes multiple primary cancers that originate from different tissues and group III consists of cancers from different tissues and organs that concurrently exist with group I cancers, and they form multiple primary cancer of three or more cancers. Group I is further subdivided into group A, which includes cancers that occur in the same tissue and organ, group B, which includes cancers that are from the same tissue and different organs, and group C, which includes cancers that occur in bilateral organs. Multiple primary cancers are again classified as synchronous and metachronous. Those malignancies that are observed at the same time or within 6 months are termed as synchronous multiple primary cancers, and those cancers that develop at more than a 6-month interval are termed as metachronous multiple primary cancers.[4] On the other hand, many studies have defined 1 year as the dividing time of these two types of multiple cancers.[5]

In Indian literature, scant data is available regarding multiple primaries, most of them being case reports, including two from our institute.[6-13] In this retrospective study, we have analyzed the multiple cancers encountered in the department of surgical oncology of a single institute over a 5 year study period.

Materials and Methods

In this retrospective study, from May 2007 to May 2012, total thirteen cases of multiple malignancies diagnosed histopathologically were retrieved from the archives of department of surgical oncology. Clinical data were obtained from the medical records. We did not include leukemia as 2nd malignancy. Furthermore, autopsy data was not included. We have not included those cases where the possibility of the 2nd malignancy being a metastatic deposit was not completely excluded. We have categorized the malignancies as synchronous if the interval between development of them was less or equal to 6 months and if it was more than 6 months we have termed it as metachronous. Positron Emission Tomography- Computed Tomography (PET-CT) was not done in any of these cases due to financial constraints.

In this retrospective study, the patients and their relatives have given consent to utilize the information for publication purpose as noted from the standard case sheet record obtained from the medical records department. As the study had no intervention other than standard care, we have not obtained permission from the institutional review board.

Results

We retrieved a total of thirteen cases in the 5 year study period. Out of them, two were in the metachronous category owing to interval between detection of primary and 2nd malignancy being more than 6 months. The synchronous ones were detected simultaneously either at the time of clinical examination or reported in histopathological examination of the surgical specimen.

There was female predominance with age range being 43-68 years. Majority of the cases were in 7th decade. The most common organ involved was breast, followed by cervix.

In the metachronous category, there were two cases. In the first case, the first primary was (IDCC (nos)) Infiltrating duct cell carcinoma (not otherwise specified) of breast and the 2nd malignancy was endometrial adenocarcinoma. This patient was diagnosed as a case of IDCC (nos) after lumpectomy which was carried out in an outside center. After that the patient did not receive any chemo or radiotherapy. She presented to our institute after a gap of 39 months with fine needle aspiration (FNA) findings suggestive of recurrence of IDCC (nos). At that time she complained of bleeding per vaginum for which she was evaluated and ultrasonography showed thickened endometrium. The patient underwent right modified radical mastectomy (MRM) for recurrence of IDCC and was advised chemotherapy and hormonal therapy. She also underwent radical hysterectomy and the final histopathological impression was endometrial adenocarcinoma.

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The 2nd metachronous case was a 37-year-old male patient in whom the first primary was detected as squamous cell carcinoma of penis. This patient was treated with emasculation, bilateral ilioinguinal block dissection and was referred to radiotherapy. However, the patient was lost to follow-up and presented again after a gap of 22 months. At that time, the routine chest X-ray showed a cavitary lesion in the left upper zone of lung. FNA of the lesion in the lung was proved to be adenocarcinoma of lung.

In the synchronous category, there were eleven cases. Out of them, four were bilateral carcinomas of breast. One bilateral carcinoma of breast revealed a histology of infiltrating lobular carcinoma and the rest that of IDCC (nos).These patients underwent bilateral MRM and received chemotherapy and hormonal therapy (Adriamycin + Cyclophosphamide for 6 cycles and Tamoxifen).

One case of bilateral carcinoma breast was detected simultaneously with endometrial adenocarcinoma of uterus.

In one case of IDCC breast, at the time of routine examination, cervical growth was detected. This patient underwent right MRM and radical hysterectomy. She also received chemo and hormonal therapy.

A breast lump was detected on the routine examination of a case of papillary carcinoma of thyroid, which later on proved to be IDCC (nos). She underwent total thyroidectomy and right MRM followed by chemotherapy.

In another case, the cervical biopsy distinctly showed adenocarcinoma of endocervix and cervical intraepithelial neoplasia (CIN-III) of ectocervix. This patient was advised radical hysterectomy but subsequently she was lost to follow-up.

Again in another case of squamous cell carcinoma of buccal mucosa, cervical growth (stage III B) was detected. This patient was referred for chemoradiotherapy.

Other cases, which were included in the synchronous variant from the department of surgical oncology were cervix showing changes of CIN-III and sertoli and leydig cell tumor of ovary and another case of squamous cell carcinoma of esophagus and mixed epithelial tumor of ovary, previously reported from our institute.[7,8]

Follow-up data was available for various patients ranging from 6 to 42 months [Table 1].

Discussion

Though, multiple primary cancers are not common, yet it is believed that the incidence is increasing. Since in patients with multiple cancers, the focus is mainly on the primary disease, there is a higher likelihood of missing incidental co-existence of another primary malignant lesion. Therefore, it is important to make an early diagnosis and administer prompt therapy in case of multiple cancers.[2]

The theory regarding the origin of majority of multiple primary cancers is that they arise as a result of random chance, but different mechanisms have been suggested to be involved in multiple primary cancers, such as the family history, immunologic and genetic defects, prolonged exposure to carcinogens, radiation and chemotherapy for the primary cancer, and field cancerization.[1,6,14,15] Previously reported cases of multiple primary cancers are mainly described in the respiratory, gastrointestinal, and genitourinary systems.[16] One autopsy series has reported prostate cancer as one of the most common malignancies in patients with multiple primary cancers and also as a frequent incidental autopsy finding in elderly men.[17]

In our study, we have encountered two cases of malignancies in the metachronous category, where the primary was carcinoma breast in one case followed by endometrial carcinoma and the other one was carcinoma penis followed by adenocarcinoma of lung.

Breast cancer patients often develop a 2nd primary malignant tumor; common sites being opposite breast, endometrium and ovary with rare primary cancer of cervix.[9]

We have noted eight cases of carcinoma breast, out of them four being bilateral. According to the classification by Moertel, they fall under the Group IC category.[3] However, Tan *et al.* did not include bilateral carcinoma breast in their study stating that they are fairly common.[18]

In our study, there was one case of synchronous bilateral breast carcinoma and endometrial adenocarcinoma.

There were also three cases of carcinoma breast and endometrial adenocarcinoma. One of the cases was metachronous and the other two were was synchronous in nature. One of the synchronous cases had bilateral IDCC (nos) breast and endometrial adenocarcinoma. However, in one case, the patient did not give any history of treatment after excision of breast lump and subsequently had endometrial carcinoma. We could not find an association between tamoxifen use for breast carcinoma and subsequent development of endometrial adenocarcinoma, though in literature it is described that tamoxifen use of at least 60 months is associated with high risk uterine histological subtypes when compared to no tamoxifen use.[19]

We also had a synchronous case of breast carcinoma and cervical carcinoma.

Goto, *et al.* in their article have described a case of synchronous invasive squamous cell carcinoma and clear cell adenocarcinoma of endocervix. They also detected Human papillomavirus (HPV) 18 in the squamous cell carcinoma; but not in the clear cell adenocarcinoma.[20] In our case the cervical biopsy showed adenocarcinoma of endocervix and CIN-III of ectocervix, but the patient was lost to follow-up prior to complete evaluation.

There was also an unusual case of synchronous papillary carcinoma of thyroid and breast IDCC.

The numbers of patients with multiple cancers have recently been increasing. In the present scenario, the possibility of a 2nd or 3rd malignant lesion should be considered for patients with primary cancer. Furthermore, the importance of screening procedures should be emphasized for the early detection of malignancy before the appearance of clinical symptoms.

In our study though, we have encountered a good number of multiple cancers in a relatively short period, most of them being synchronous, still a larger multi-institutional study with longer follow-up is required to arrive at a definite conclusion regarding the true incidence of multiple primaries.

Clinical characteristics

			First primary and histology	Treatment	Time interval	Second primary and histology	Treatment	Follow-up
			Breast, IDCC (ase)	Right mastectomy No RT, no CT	59 months	Uterus Endometrial adenocarcinoma	A. Right MRM for recurrence, CT, Letrozole/patient refused RT B. Radical hysterectomy	5 months
			Penis, SCC	Emasculation, BA, IBD referred to RT, lost for follow-up	33 months	Lung, primary adenocarcinoma	Advised palliative chemotherapy. Again LFU	-
5	50/F	Syn	BA, advised ILC	-	-	-	BA, MRM	12 months
6	48/F	Syn	BA, breast IDCC	-	-	-	Neoadjuvant CT-4 cycles, Bilateral MRM, Completion CT, HTa	39 months, No recurrence
7	55/F	Syn	BA, Ca breast at after 6 month left, IDCC (ase)	-	-	-	CT-AC4 cycles BA, MRM, Completion CT	42 months No recurrence
8	45/F	Syn	A. BA, ca breast, IDCC (ase) B. Uterus Endometrial adenocarcinoma	-	-	-	A. BA, MRM, CT (8 cycles) B. Radical hysterectomy	39 months No recurrence
9	60/F	Syn	A. Breast, IDCC (ase) B. Uterus Endometrial adenocarcinoma	-	-	-	A. Left MRM, CT (4 cycles+HTa) B. Radical hysterectomy	9 months
10	60/F	Syn	A. Thyroid, PTC B. Breast, IDCC (ase)	-	-	-	A. Total thyroidectomy B. Right MRM CT (AC 4 cycles+ HTa)	12 months
11	65/F	Syn	A. Breast, IDCC (ase) B. Cervix, SCC	-	-	-	A. Right MRM+CT (FAC 5 cycles+Tamoxifen) B. Radical hysterectomy	24 months No recurrence
12	60/F	Syn	Cervix Carcinoma in situ (advocervix) Adenocarcinoma (endometrium)	-	-	-	Advised radical hysterectomy	-
13	60/F	Syn	A. Buccalmucosa, BCC B. Cervix, SCC	-	-	-	Chemo RT	8 months
14	45/F	Syn	A. Cervix Carcinoma in situ (CIN-III) B. Ovary-sarcoid and lipoid cell tumor	-	-	-	Radical hysterectomy Planned for CT and RT	7 months
15	50/F	Syn	A. Esophagus-SCC B. Ovary, mixed epithelial tumor	-	-	-	A. Palliative gastrostomy followed by radiotherapy B. Excision of ovarian cyst	

Abbreviations: Clinical examination, CT=Chemotherapy, RT=Radiotherapy, BA=Abdominal hysterectomy and salpingo-oophorectomy (unless otherwise specified), SCC=Squamous cell carcinoma, PTC=Papillary carcinoma of thyroid, AC=Adriamycin+cyclophosphamide, FAC=5-Fluorouracil+adriamycin+cyclophosphamide, IBD=Ileo anal pouch anastomosis, Mets=metastasis, Syn=synchronous, BA=Abdomen, HTa=Hormone therapy

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Original Article

Androgen Receptor Expression in Triple Negative Breast Cancer - Study from a Tertiary Health Care Center in South India

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Abstract:

Background: The treatment of breast cancer is based on a multi-modality approach. Analysis of the hormone receptor has been accepted as a standard procedure, in the routine management of breast cancer patients. Triple negative breast cancers (TNBCs) are those which are negative for expression of all three markers, i.e., estrogen receptor, progesterone receptor (PR), and human epidermal growth factor receptor. High expression of Ki-67, a proliferation index, has been associated with a worse prognosis in TNBC. TN cancers are aggressive in nature as they do not respond to routine targeted therapy. The role of the androgen receptor (AR) in breast carcinomas is important as AR has been suggested as a potential therapeutic target. We did this study to assess AR immunoreactivity in TNBCs and correlate with Ki-67 index. **Materials and Methods:** In this study group, there were 45 cases of TN invasive breast carcinomas. These tumors were analyzed with respect to AR and Ki-67 index. **Results:** Of 45 TN breast carcinomas analyzed, 42 were infiltrating duct cell carcinoma (IDCC) of not otherwise specified type. There were one medullary and two metaplastic carcinomas. The median age was 46 years. AR was positive in 21% (9/45) of cases. All the AR positive cases were an IDCC (rest). Out of the nine AR positive cases, six showed positivity for Ki-67. The statistical analysis using Pearson's Chi-squared method did not reveal any correlation between AR and Ki-67 index ($P = 0.574$). **Conclusion:** Although our study did not reveal any statistically significant correlation between AR and Ki-67 index, assessment of AR status in TNBC patients is desirable as it may help to develop a targeted therapy in future in these aggressive cancers.

Keywords: Androgen receptor, breast cancer, Ki-67 index, triple negative breast cancer

Introduction

Breast cancer is one of the most common malignancies in females worldwide.[1] The incidence of breast carcinoma is rapidly increasing in India. Currently, according to the International Agency for Research on Cancer, breast cancer is the most common cancer in Indian females.[2] The treatment of breast cancer is based on a multi-modality approach.[1] Analysis of the hormone receptor has been accepted as a standard procedure, in the routine management of patients with breast cancer. Triple negative breast cancers (TNBCs) are those which are negative for expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor (HER-2 neu).[3-5] TN is considered to be an aggressive form of breast cancer as they do not benefit from the standard targeted therapies.[3-5] High expression of Ki-67, a proliferation index, has been associated with a worse prognosis in TNBC.[6]

The role of the androgen receptor (AR) in breast carcinomas has drawn great attention in recent years, especially due to its expression in ER and PR negative breast carcinomas.[7-9] AR has been suggested as a potential therapeutic target.[10]

In this study, we have included consecutive TN cases for 1 year and attempted to correlate with AR receptor status and Ki 67 levels.

Materials and Methods

There were 148 cases of TN breast carcinomas (negative for ER, PR, and HER-2) in 10 year study. The criteria for determining triple negativity were based on immunohistochemical (IHC) staining. In TNBC patients, ER and PR staining were 0% by IHC, and HER2 staining was 0 by IHC or 1+.

In the present study, 45 consecutive cases of TN breast carcinomas, diagnosed in 1 year period, were retrospectively analyzed. In

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...in breast carcinoma cases, immunostaining for ... time. This study was conducted in a single tertiary ... institution from south India, We have excluded breast ... cases from other health care centers, where ... paraffin blocks of were received only for review and IHC ... analysis due to unknown fixation status. *In situ* carcinomas ... and cases with incomplete information were also excluded from the study. The in-house specimens (modified radical mastectomy, lumpectomy, excisional biopsy, tru-cut) were fixed by 10% neutral buffered formalin were included in the study group. The histopathology and IHC reports (ER, PR, HER-2, and Ki-67) were accessed from the computerized hospital information system. AR immunostain was done on 3 μ paraffin sections on 3-amino propyl ethoxysilane coated slides. Antigen retrieval was done by pressure cooking for 5–10 min in Tris EDTA buffer, pH 9.0. IHC was done by Polymer HRP (Horse Radish Peroxidase) IHC detection system. Primary antibody used for AR staining was monoclonal mouse anti-human antibody (clone-F 39.4.1). Prostate carcinoma case was taken as positive control for AR. The clone used for Ki-67 was BGX-Ki67. The antibodies used were prediluted. All the markers were from BioGenex [Table 1].

The slides were stained with 3, 3'-diaminobenzidine tetra hydrochloride chromogen, counterstained with hematoxylin and mounted.

Tumor expressing 10% or more nuclear positivity for AR was considered positive. For each case, five hundred cells were counted to calculate the nuclear positivity for Ki-67. Two hundred cells were counted in the case of tru-cut biopsies. Ki-67 was considered to be positive when the nuclear positivity was more or equal to 10%[15] [Figures 1 and 2]. The normal epithelial elements served as an internal control.

The data obtained was analyzed using the SPSS 11.5 (Chicago, IL., USA) statistical program. Pearson's Chi-squared method was used to obtain a correlation between AR and Ki-67.

Results

A total of 45 TN breast carcinomas were analyzed. There was one medullary and two metaplastic carcinomas among them, rest being infiltrating duct cell carcinoma (IDCC) not otherwise specified (IDC (nos)). The age range was wide (26–76 years). The median age was 46 years. AR was positive in 20% (9/45) cases. The AR positive cases were all IDCC (nos). Out of the nine AR positive cases, six showed high expression for Ki-67. The statistical analysis using Pearson's Chi-squared method did not reveal any correlation between AR and Ki-67 index ($P = 0.574$).

Discussion

There is a sharp rise in breast cancer worldwide. Breast cancer is a leading cause of cancer death in the less developed countries of the world.[5] India also is witnessing an alarming rise of breast cancer.[6] ER and PR have gained widespread acceptance as independent prognostic parameters in breast carcinoma. Now a days, a standard work-up for breast carcinoma includes evaluation of ER, PR, and HER-2/neu status for therapeutic consideration. TN tumors are those which are negative for ER and PR as well as for HER2/neu. These tumors represent approximately 25% of all breast cancers.[7-9]

In a previous study of breast carcinomas in 10 year study, we had observed 22.7% TN cases. However, there was no significant correlation between these TN cases and parameters such as age, tumor size, tumor grade, lymphadenopathy, and p53.[8]

The AR is expressed in normal breast tissue, its expression decreases as there is progression to *in situ* ductal carcinoma

Table 1: Source and dilution of antibodies

Antigen	Antibody	Clone	Manufacturer	Dilution
AR	Monoclonal	F 39.4.1	BioGenex	Prediluted
Ki-67	Monoclonal	BGX-Ki-67	BioGenex	Prediluted

AR – Androgen receptor

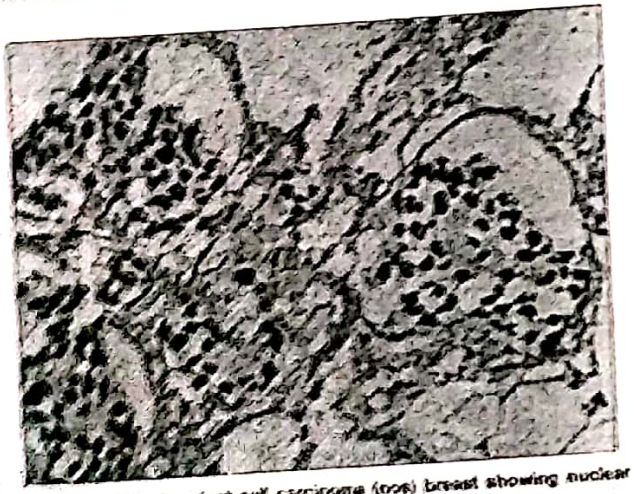

Figure 1: Infiltrating duct cell carcinoma (nos) breast showing nuclear positivity for androgen receptor (immunohistochemistry, ×200)

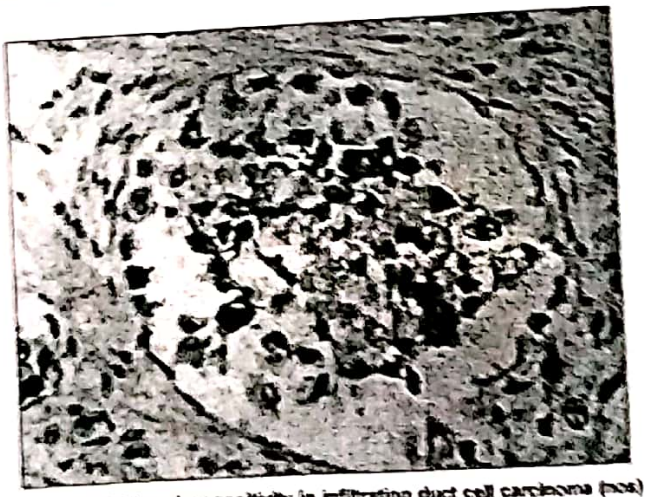

Figure 2: Ki-67 nuclear positivity in infiltrating duct cell carcinoma (nos) breast (immunohistochemistry, ×400)

and invasive cancer.[9] AR is a member of the steroid hormone receptor family and is implicated in breast cancer pathogenesis. The current theory suggests that in breast carcinogenesis, the androgen signaling pathway plays a critical role independent of ER.[1,4,10] AR expression in normal luminal mammary epithelial cells is approximately 20%.[11] However, 70% of invasive and intraductal breast cancers express AR.[1,4,12,13] A significant number of poorly differentiated breast carcinomas which are negative for ER and PR, are positive for AR.[11]

Recently, there is increasing interest regarding the role of the AR, particularly in patients with TN tumors. TN tumors generally have a more aggressive clinical course and do not benefit from conventional endocrine targeted therapies. However, recent evidence suggests that there may be role for AR as a therapeutic target for a subset of TNBC.[1,4-6]

One study from India by Sharma *et al.* noted 31.9% TNBC cases. They found that these cases present in younger females are associated with high grade, large tumor size, and high rate of lymph node positivity. The most common histological subtype in TNBC was IDC (nos).[3] In our study also most of the TN cases were IDC (nos). We found nine (20%) TN cases to be positive for AR. Most of the IHC studies have found the AR positive tumors represent a small subset within TNBCs, ranging from 12% to 23%.[14,15] In a large study of over 2,000 invasive breast cancer, AR positivity was reported in 32% of TNBC.[14] Sutton *et al.* observed that in 31.4% of TNBCs there is a positive expression of AR.[5] McGhan *et al.*, have described AR positive TN tumors to be more common in older patients, prone for lymph nodal metastasis and more advanced disease.[9] Whereas, Sutton *et al.* noted that AR positive TNBCs are less likely to have distant metastasis.[5]

Bicalutamide is an oral, nonsteroidal, and AR antagonist.[14] A recently completed phase II trial of Bicalutamide in advanced TNBC involved a prospective screening step, in which TNBC tumors were assessed for AR expression before being assigned to therapy. The frequency of AR positivity by IHC was low (12%). This trial reported a clinical benefit rate of 19% and a median progression free survival of 12 weeks.[15]

Ki-67 is a proliferative marker with the highest expression during mitosis. Ki-67 is used as a prognostic marker in breast cancer. Higher expression of Ki-67 is associated with worse prognosis. In TNBCs, higher Ki-67 expression is associated with worse prognosis. Sutton *et al.*, observed a negative correlation between AR and Ki-67 expression. Hence, they opined that since AR positive tumors have lower Ki-67 index, high levels of AR may be associated with better prognosis in TN carcinomas. They have attributed this lower expression of Ki-67 in AR positive tumors, to the antiproliferative effect of AR.[5] However, we did not observe any statistically significant correlation between AR and Ki-67 expression in TNBCs, in our study.

Conclusion

In the current study, a subset of TNBCs (20%) are positive for AR. Antiandrogen therapy may be tried in those TNBCs expressing AR as the TN cancers do not respond to standard targeted therapy and are aggressive in nature. We did not find any significant correlation between AR and Ki-67 expression. However, results from multi-institutional studies with better sample size and follow-up data should be analyzed before advocating anti-androgen therapy for TNBCs showing AR positivity.

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Conflicts of interest

There are no conflicts of interest.

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