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AUTOMATED QUANTITATION OF RECEPTOR STATUS OF BREAST CARCINOMA WITH IMMUNOHISTOCHEMICAL RECLASSIFICATION

P. Aruthra[1], R. Vijayashree[2], K. Ramesh Rao[3]

ABSTRACT: BACKGROUND AND PURPOSE: Recent developments in our understanding of biology of breast cancer and introduction of molecular methods have resulted in a new molecular classification. However, these methods are expensive. But, these molecular groups can be roughly identified for practical purposes as Surrogate Molecular Classes by relatively inexpensive immunohistochemistry (IHC) using antibodies directed against ER, PR and HER2. We have tried to assess the Immunohistochemical profiles of the carcinomas of the breast that we received in our laboratory; while doing so, we have used Immuno Ratio and Immuno Membrane image analyser software to accurately assess staining reaction. We have used these profiles to reclassify according to the new molecular classification (Surrogate Molecular Classes) and correlated the same with morphological types and projected outcomes derived from risk predicting protocols. **MATERIALS AND METHODS:** 35 cases of breast carcinomas were analysed immunohistochemically for the demonstration of expression of oestrogen receptor (ER), progesterone receptor (PR), HER 2 and cytokeratin (CK). The immunohistochemical reaction was quantified using Immuno Ratio and Immuno Membrane online softwares. The results were used to reclassify according to the new molecular classification. We have also correlated the same with morphological types and projected outcome derived from risk predicting protocols. **OBSERVATIONS AND CONCLUSIONS:** Automated immunohistochemical quantitation revealed that Estrogen receptor (ER) and Progesterone receptor (PR) were positive in 94% of cases. There were two triple negative cases. When we reclassified the cases based on the immunological profile obtained from this analysis, Luminal A type was found to be the commonest type. There were two basal types which had the worst projected survival rate. Immunoratio is useful and easy to use free online grading software. However it needs to be calibrated and used in advanced mode to obtain optimal results.
KEYWORDS: Breast Carcinoma, Cytokeratin (CK), HER 2, Immuno Ratio, Immuno Membrane, Oestrogen Receptor (ER), Progesterone Receptor (PR).
MESHTERMS: Breast neoplasms/ Classification; Cytokeratin Antigen; Receptor, ERBB2; Receptors, Estrogen; Receptors, HER2; Receptors, Progesterone.

INTRODUCTION: Carcinoma of breast is not a single disease morphologically. It consists of many entities that differ histologically. These histological variations influence the clinical presentation, biologic behaviour and therapeutic response to a varying extent. Based on these morphological variations, several classifications have been developed over the years to guide the clinician in therapeutic management of these lesions. The latest of these is 2003 edition of WHO classification.[1] Morphological classification is easy to implement and fairly reproducible and are popular in all the laboratories that report on breast cancer.

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However, some of the recent developments in our understanding of the biology of breast carcinoma have exposed the shortcomings of these classifications. First of these developments is the recognition of receptor expression by these tumours which could be targeted for hormonal/adjuvant therapy [2] .The second is the realization that these tumours are heterogeneous at molecular level as well and this heterogeneity is responsible for its biologic behaviour and response to therapy. Third is the advances in breast conservative surgery that forced the surgeon to demand more accurate prognostic prediction from the pathologist. The traditional morphological classification is inadequate to meet these new challenges.

Around the turn of the century, several studies using microarray technology recognized biologically distinctive groups at molecular level within carcinoma of breast. This grouping reflected the biological behaviour of the tumours and could be used to predict outcome and therapeutic response. A new molecular classification was developed based on the expression of 5 molecular markers: ER, PR, HER2/neu, CK5/6 and EGFR. It consists of four subtypes: LUMINAL A, LUMINAL B, HER-2 enriched type and BASAL subtype[3,4] Soon it was discovered that these molecular groups could be roughly identified for practical purposes as Surrogate Molecular Classes by relatively inexpensive immunohistochemistry (IHC) using antibodies directed against ER, PR and HER2.[5,6] Since then, IHC for these markers is being widely used in the management breast cancer due to availability of cost effective simple Immunohistochemical kits.

To ensure the accurate assessment of positive IHC reaction, it has become necessary to grade it. There are several protocols available to achieve this. One such protocol has automated the process and is available freely online. It is called Immuno Ratio/ Immuno Membrane.[7]

In the present study, we have tried to assess the Immunohistochemical profiles of the carcinomas of the breast that we received in our laboratory from 2012 to 2014; while doing so, we have used Immuno Ratio and Immuno Membrane image analyser software to accurately assess staining reaction. We have used these profiles to reclassify according to the new molecular classification (Surrogate Molecular Classes). We have also correlated the same with morphological types and projected outcome derived from risk predicting protocols.

MATERIALS AND METHODS: The present study was carried out in the department of Pathology, CHRI, Chennai, South India, on 35 cases of breast carcinoma received in the laboratory. Specimens received in 10% buffered formalin were grossed, appropriately sampled and processed for paraffin embedding as per the recommended guidelines.[8,9] From each block at least 5 sections were cut at 3μ thickness. These sections were used as follows:

- One section was stained with routine Hematoxylin and Eosin (H&E). H and E stained sections were examined microscopically to establish morphological diagnosis, histological subtyping and grading of the tumours. The grading of the tumours was done according to Nottingham modification of Scarff, Bloom & Richardson Grading (NGS).[10]
- Other four sections taken on poly-L-Lysine coated slides were used for immune-histochemical (IHC) demonstration of Estrogen Receptor (ER), Progesterone Receptor (PR), ERBB2 (HER-2) and Cytokeratin Cocktail (CK 5/7/8/18) using Novocastra™ mouse monoclonal antibodies. High temperature antigen retrieval ABC method recommended by Leica was employed for IHC. [11] Sections stained with IHC for ER, PR, HER2 and CK were microscopically examined, and regions of interest were digitally photographed at 200X using Olympus SP350 (8 MP) camera

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and Olympus CX 41 microscope and saved as jpeg images. The intensity of staining reaction of ER and PR were scored by uploading the images to online Immuno Ratio software (http://153.1.200.58:8080/immunoratio/); Immuno Ratio scores the intensity and extent of staining and expresses the result as a percentage. ASCO/CAP recommend ≥1% staining of tumor nuclei as a cut-off between positive and negative reaction. HER2/ CK were analysed by Immuno Membrane (http://153.1.200.58:8080/immunomembrane/) software. HER2 and CK are graded by Immuno Membrane into three grades: 0/1, 2 and 3. Only 3+ reaction was taken as positive as per ASCO/CAP guidelines. The result output of both immunoratio and ImmunoMembrane is a montage of original IHC reaction and the analysed one. (Fig 1, 2, 3, 4)

The Nottingham Prognostic Index (NPI) was calculated online using the following formula (http://primed.info/finprog/npi_expl.asp):[12]

- Tumor size in cm x 0.2 + lymph-node stage (1, 2 or 3) + histologic grade (1, 2 or 3).

The scoring was done as shown in table 1:

Excellent Prognosis	<= 2.4
Good Prognosis	<= 3.4
Moderate Prognosis	> 3.4 and <= 5.4
Poor Prognosis	> 5.4
Table 1: NPI Index Score	

St. Gallen 2001 Consensus Criteria [13] was applied to stratify the cases into low risk and average-high risk categories. Adjuvant! For breast cancer online version 8 (https://www.adjuvantonline.com/online.jsp) was used to determine the 10 year mortality/ survival rate.

The cases were reclassified as follows based on the status of expression of the four markers used in the study, namely, ER, PR, HER2 and CK (table 2):

Marker expression criteria	Class
ER + and/or PR +, HER 2 negative, CK +	Luminal A
ER + and/or PR +, HER 2 +, CK +	Luminal B
ER -, PR -, HER2 -	Triple negative/Basal
ER -, PR -, HER2 +	HER Enriched type
Table 2: Immunohistochemical Classification	

The results were statistically analysed where required using SPSS software version 21. Independent student t test was performed and p value of <0.5 was accepted as significant.

RESULTS: CLINICAL FEATURES: The age of the patients ranged from 30 to 85 years with a mean age of 48.80 (SD±10.64). Nearly two-thirds of the patients were in the age group of 41-50 years. 21 of these patients (62%) were premenopausal. The most common clinical presentation was lump in the breast with or without pain (34/35). Only other clinical manifestation was discharge without a palpable mass which was observed in only one case. Left breast was affected in nearly three-fourths

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of cases (74.28%). Most common location within the breast was upper outer quadrant (54.28%) followed by upper inner (20%) and central retro-areolar region (14.28%).

MORPHOLOGICAL TYPES AND GRADING: The ductal carcinoma was overwhelmingly the predominant morphological type in this study accounting for 94.4% of cases (33/35). The other two cases were invasive lobular carcinoma. Within the ductal group, more than two-thirds of the cases were invasive ductal carcinoma (NOS) (68%). Majority of these patients (14/24; 58.3%) were in 41-50 age group. Other subtypes were 5 cases of ductal carcinoma with medullary like features (14.2%), three cases of poorly differentiated carcinoma (8.57%) and one case of mucinous carcinoma (2.8%).

When the tumours were graded by NGS criteria, 60% of the tumours (21/35) were found to be grade 3 tumours. Of the rest, 28.14% (13/35) were grade 2 tumours. Only one case was found to be in grade 1. All the three poorly differentiated tumours occurred in older women (p = <0.02). Only four cases showed lymph node involvement.

PROGNOSIS AND RISK ASSESSMENT: When the NPI was calculated, 71.42% of cases were in moderate prognosis group (25/35). Most of these patients were in 41-50 age group. None of the cases were in excellent prognosis group. When NPI was applied to individual tumours, it was found that majority of the NOS were high grade tumours (16/24; 66%) belonging to moderate prognosis group (19/24; 79.16%).

When the risk of negative outcome was assessed through Adjuvant! Online, poorly differentiated carcinoma had significantly lower projected survival rate compared to other morphological forms (p<0.05).

IMMUNOHISTOCHEMISTRY (IHC): Results of the immunohistochemistry are summarized in the table 3 along with probable molecular type. The results are interpreted as per the recommendations of ASCO/CAP. The cut-off percentage for a positive result for ER and PR were kept at 1% staining of the tumour nuclei:

- ER+/PR+ Cases: Both ER and PR were positive in 33 cases (Fig 1 and 2). The percentage of positivity varied from 1% to 100% for ER and 1.3 to 100% for PR. Twelve of these cases also showed 3+ HER2 positivity (34.28%). In two cases, both ER and PR recorded values less than 1 percent. In these two cases HER2 was also negative or weakly stained. So these two cases were triple negative. Only CK gave strong (3+) positivity. There were no ER/PR negative cases with HER2 positivity.
- HER2 + cases: A staining reaction of 3+ was taken as positive result as per ASCO/CAP guidelines (Fig 3). HER 2 was positive in 12 cases but all of them were also positive for ER and PR. There were no ER-/PR- cases that were positive for HER2.
- CK: CK was positive in all the cases. In 25 cases, it gave intense reaction (3+) (Fig 4) while in others the reaction was 2+.

The results of the IHC were used to reclassify the cases as follows (Table 3):

Marker expression criteria	Class	No. of cases	%	Age group
ER + and/or PR +,	Luminal A	21	60	37-82

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HER 2 -, CK +				(48.28 ±10.78)
ER + and/or PR +, HER 2 +, CK +	Luminal B	12	34.28	35-63 (47.33±7.44)
ER -, PR -, HER2 -	Triple negative/Basal	2	5.71	44±1
ER -, PR -, HER2 +	HER Enriched type	0	0	
Table 3: Results of IHC with Immunohistochemical Classification				

Most of the cases in our study belonged Luminal A type (21/35; 60%), with average age incidence of 48.28%. Luminal 8 accounted for 12 cases (12/35; 34.27%). The average age of this group was marginally less than the previous group. The remaining two cases belonged to basal type which occurred in slightly younger individuals. There were no cases of HER2 in our series.

When morphological types were compared to the surrogate molecular subtype, it was found that 61.9 % (13/21) of luminal A and 75% (9/12) of luminal B and both cases of basal type were morphologically NOS type. When risk for negative outcome was assessed, it was found that patients with basal were projected to fare much worse than the other types (SR of 47.5 vs 70; $p<0.01$). Survival rates for Luminal A and luminal B were similar (71.6 & 70). But the mortality rate was the lowest for Luminal A.

When the molecular subtypes were assessed by NPI, majority of the patients in all the groups were in moderate to poor prognosis category (Luminal A 18/21, 85.7%; Luminal B 10/12, 83.3% and both cases of basal type)

DISCUSSION: In the present study involving 35 cases of carcinoma of the breast, the average age of the affected patients was 48.80 (±10.64) years. Sixty-two percent of those were premenopausal. Five of the patients were less than 40 years old. The left upper outer quadrant was the most commonly affected site (74.28%). The most common presentation was a lump in the breast with or without pain (34/35; 98%). Similar observations with regard to age incidence were made by Raina et al [14], SIRO [15] and Munjal et al. [16]

The predominant morphological type in our series was invasive ductal carcinoma (IDC NOS) which formed 68.5% of all cases (24/35). It was followed by invasive ductal carcinoma with medullary features (5/35; 14.2%). Invasive lobular carcinoma was uncommon (2/35; 5.71%). Nearly two-thirds of cases were grade 3 tumours (21/35; 60%). There was only one tumour with grade 1 features. This agrees with the observations in SIRO report, [15] in which IDC NOS is mentioned as the commonest type (88%) with lobular carcinoma accounting for 3.7% of cases. Seventy percent of patients in their series had grade III carcinoma. Munjal et al [16] in their study on 107 cases of breast carcinoma, also found infiltrating duct carcinoma to be the predominant type (93.3%).

NPI scores in our study was greater than 3.4 but equal to or less than 5.4 in 25 cases (71.42%) indicating moderate prognosis. Most of these patients were in 41 to 50 age group and had high grade tumours. These findings are in contrast to the observations of Zuber Ahmed et al [17], who found NPI scores of greater than 5.4 in more than half of their cases (56.1%). This may be because of the difference in the stage of the disease. In our series only 4 out of 35 (10%) cases showed nodal involvement unlike their study in which most of the patients (70%) had nodal metastasis.

Both ER and PR were positive in 33 cases (33/35; 94%). The percentage of tumour cell positivity varied from 1% to 100%. This is in marked contrast to the results reported in several other

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studies from India. Most of these studies appear to suggest that number ER+ cases are lower in India than in western countries. Navnani et al [18] recorded only 23.2% ER+/PR+ cases in their series conducted at Breach Candy Hospital in Mumbai. In the same hospital, ER-/PR- cases accounted for 47% of cancer breast cases. The latter is probably due to occurrence of high grade tumours in premenopausal women. Many of these women seek medical help when the disease has progressed to an advanced stage. But this is not a uniform observation. According to one study, faulty immuno-histochemical staining technique may be responsible for low ER+ status among Indian breast cancer patients.[19]

HER2 was positive in 12 cases (34%). In all these cases ER/PR were also positive. This is much higher than the figure mentioned in SIRO report[15] in which Triple positive cases are claimed to account for around 15% of cases. Navnani et al have found unusually high positivity for HER2 in Indian women.[18] Our results agree with ASCO report[20] which observes that majority of breast cancer cases in Africa and South Asia are ER+.

All the three (ER, PR, HER2) receptors were negative in two cases (2/35; 5.71%). Only CK was positive in these triple negative cases.

When the results of the receptor expression study were applied to reclassify our cases, we found 21 cases of Luminal A, 13 cases of Luminal B and 2 Basal-like (Triple Negative) cases. There were no HER2 enriched type.

In our study, Luminal A was the predominant type accounting for 60% cases. Luminal B was the second most common type (37.1%). Basal type (triple negative) accounted for 5.7% of cases. As we did not use to a proliferative marker (Ki67) in our study to distinguish Luminal A from Luminal B, there may be a bit of overestimation of Luminal A. Besides, we did not have a single case of HER2 enriched type. This is in marked contrast to the findings of Munjal et al.[16] They found 37.7% luminal A, 11.1% Luminal B, 29% HER2 and 7.5 Basal-like in their series of 107 cases. They recorded unusually high number of HER2 cases. As far as the age incidence is concerned, Luminal A type tended to occur in slightly older individuals (Mean age 48.28) than Basal type (mean age 44). Similar observation was made by Munjal et al.

Basal-like (Triple negative) cases represent a subset of breast cancers with higher incidence in younger individuals, higher morphological grade and aggressive behaviour. In one study[21] published from Mumbai, India, triple negative (basal type) breast cancer formed more than 25% of the cases reported and it was associated with younger age, shorter relapse free survival, higher relapse rate and aggressive behaviour. We had only two cases. But they occurred in younger women and were associated with higher projected mortality rate compared to other types.

When we used St. Gallen Consensus Criteria to assess the risk, all our cases were in Average-High risk group. This is probably due to the large tumour size at presentation and high tumour grade. Adjuvant! Online showed that projected survival rate for both Luminal A and Luminal B were close to 70%. The Basal type had the lowest survival projection rate of 47% with high projected mortality of 50%.

In our study, we used two new grading softwares: Immuno Ratio and Immuno Membrane.[7] These two are automated, free, online image analysis softwares allowing objective evaluation of the extent and the intensity of chromogenic reaction. Both protocols are also available as NIH's Image J plugins for offline assessment. In the online version there are two modes: almost fully automated Basic mode and a semiautomated Advanced mode. Basic mode allows only one user input and no fine

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tuning. In advanced mode, the quality of the assessment can be fine-tuned by several user inputs. These include blank field correction, adjustment of pixel size (image scale) and threshold adjustment.

Results with the Basic mode are somewhat unreliable. Unusually high values were recorded for some nearly negative cases. Advanced mode allows for a much more accurate and reliable outcome. Consistent results are possible once the software is calibrated. In this study we did not compare with other manual grading methods. However, Sundararajan et al [22] compared manual and automated methods and found that they showed excellent correlation. As other manual methods of grading are somewhat subjective and time consuming, Immuno Ratio and Immuno Membrane provide a quick and hassle free solution. But their recommended 20% cut-off value does not agree with latest ASCO/CAP recommendations.

CONCLUSIONS: Some of the important conclusions of the present immunohistochemical study are as follows. Average age incidence of breast carcinoma in our study was 48.85 years. Infiltrating Ductal carcinoma NOS was the most common morphological type. The tumours were mostly in Grade 3. Estrogen receptor and Progesterone receptor were positive in 94% of cases. On reclassification (Surrogate molecular classification), Luminal A type was found to be the commonest type. The basal types had the worst projected survival rate. Immunoratio is useful and easy to use free online grading software. However it needs to be calibrated and used in advanced mode to obtain optimal results.
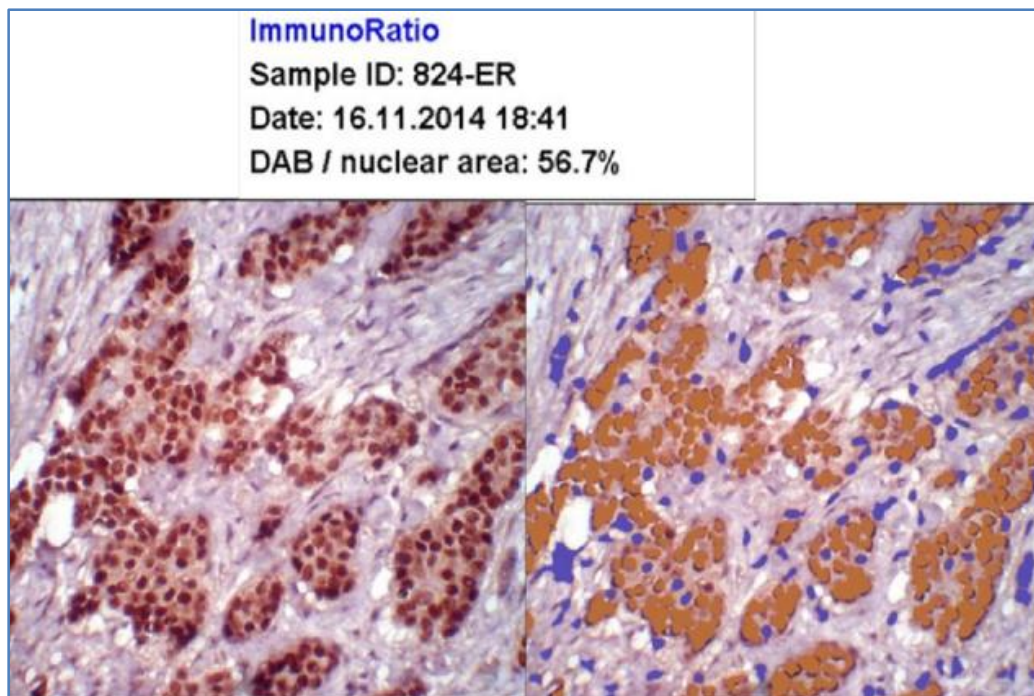

Fig. 1: Result output of ImmunoRatio for ER: a montage of original photograph of the immunohistochemical reaction and the analysed image. Score = 56.7% (Immuno Ratio output)
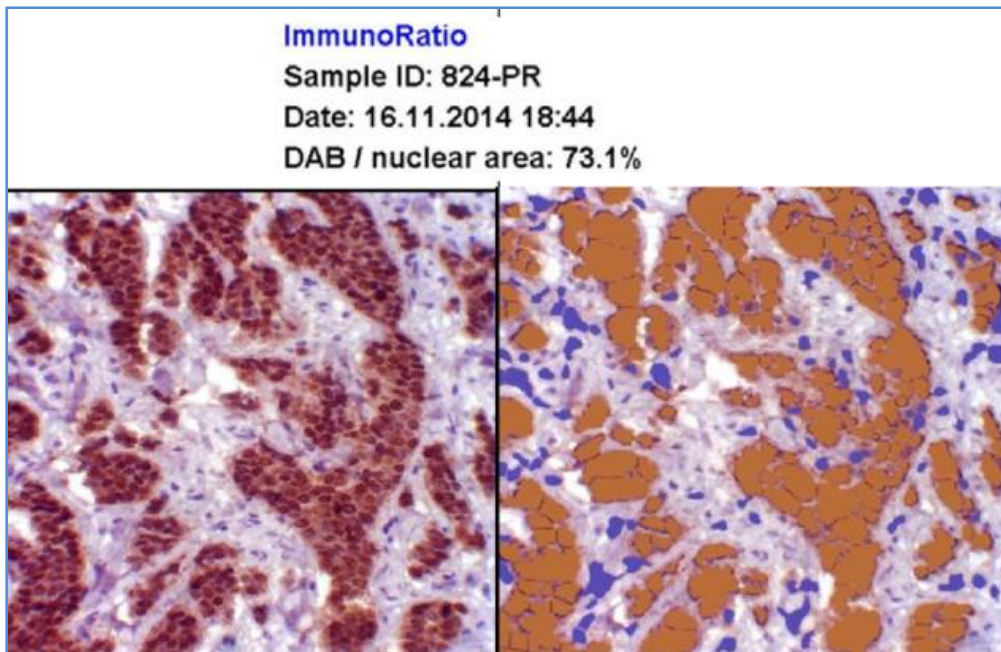
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Fig. 2: Result output of ImmunoRatio for PR: Score=73.1
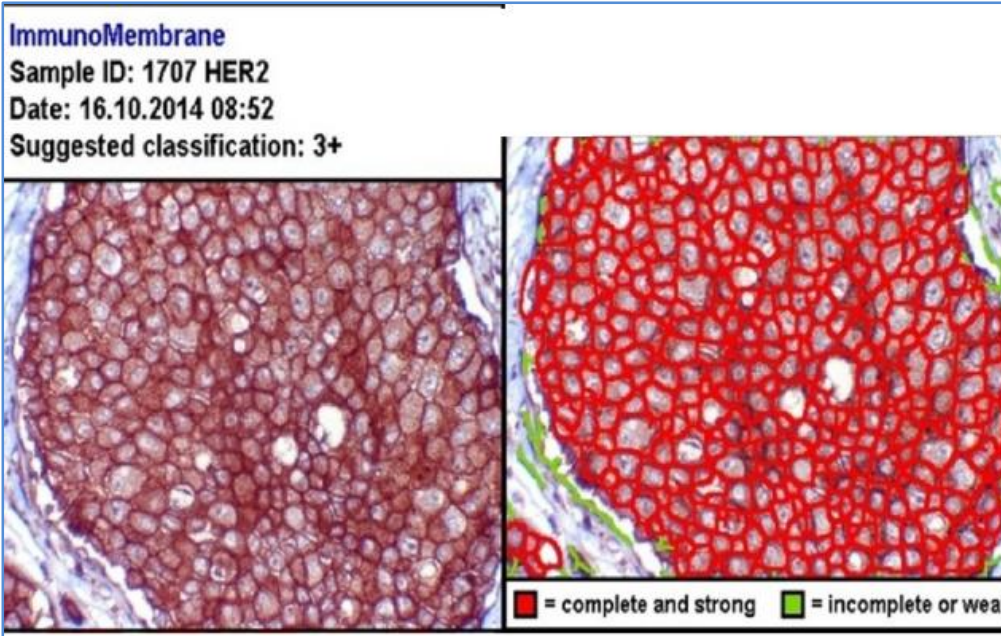
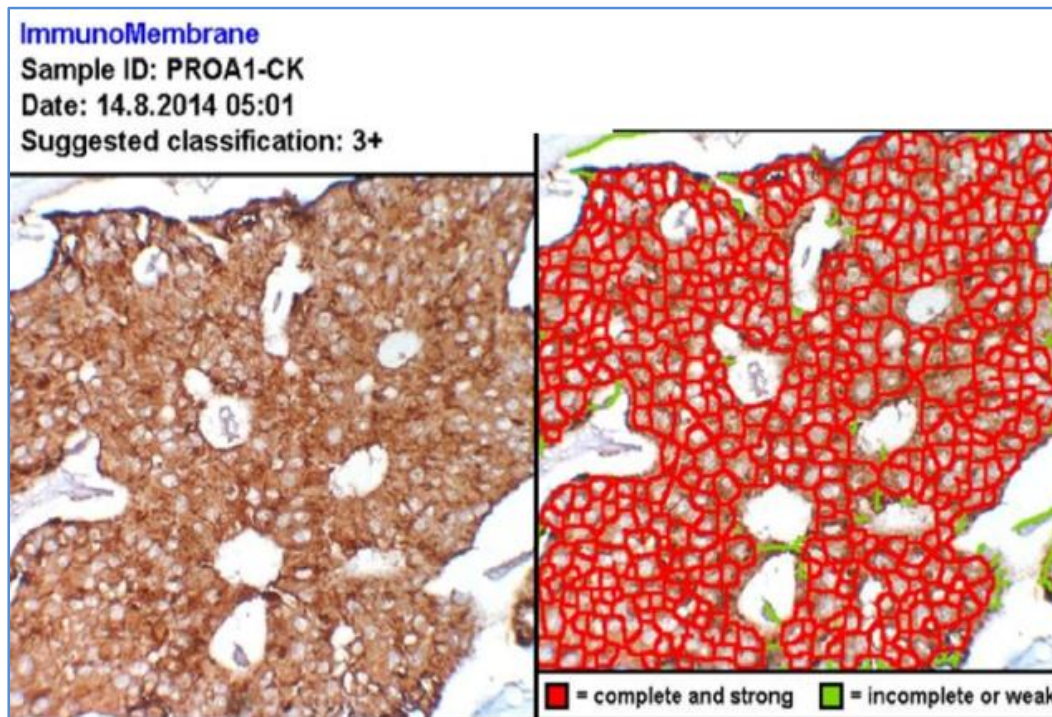**Fig. 3: Result output of ImmunoMembrane for Her 2: 3+**

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Fig. 4: Result output of Immuno Membrane for Cytokeratin (CK): 3+

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AUTHORS:
1. P. Aruthra
2. R. Vijayashree
3. K. Ramesh Rao

PARTICULARS OF CONTRIBUTORS:
1. Post Graduate Student, Department of Pathology, Chettinad Hospital & Research Institute.
2. Associate Professor, Department of Pathology, Chettinad Hospital & Research Institute.

FINANCIAL OR OTHER COMPETING INTERESTS: None

3. Professor, Department of Pathology, Chettinad Hospital & Research Institute.

NAME ADDRESS EMAIL ID OF THE CORRESPONDING AUTHOR:
Dr. K. Ramesh Rao,
Professor, Department of Pathology,
Chettinad Hospital & Research Institute,
Rajiv Gandhi Salai Kelambakkam,
Kanchipuram District-603103, Tamilnadu.
E-mail: rameshkrao@gmail.com

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