www.jmscr.igmpublication.org
Impact Factor (SJIF): 6.379
Index Copernicus Value: 71.58
ISSN (e)-2347-176x ISSN (p) 2455-0450
crossrefDOI: https://dx.doi.org/10.18535/jmscr/v6i7.16

IGM Publication
Journal Of Medical Science And Clinical Research
An Official Publication Of IGM Publication

Use of Immunomarkers D2-40 and CD31 in Detection of Lymphovascular Invasion in Breast Carcinoma

Authors
Dileep A P [1], Prasad P H[2]
[1]Junior Resident, [2]Professor
Department of Pathology, Govt. Medical College, Thrissur, Kerala, India
Email: *[1]dileeppowers@gmail.com, [2]drprasadph@gmail.com*

Abstract
Breast cancer is a major health problem in the world. It is a disease with significant morbidity and mortality. Metastasis and recurrence are the main causes of mortality in breast carcinoma. Lymphovascular invasion is associated with increased axillary node metastasis and recurrence. Identifying lymphovascular invasion (LVI) is difficult in haematoxylin and eosin stained slides especially if lumen is completely filled by tumour cells or when retraction artefacts is present. D2-40 is a sensitive and specific marker to detect tumour cells in lymphatic vessels. It selectively and specifically stains lymphatic endothelial cells. CD31 is a 130 kDa transmembrane glycoprotein in the immunoglobulin super family. CD31 stains blood and lymph vessels. Staining with these immunomarkers makes it easy to identify the invasion. If it is identified more aggressive therapy can be given to the patient which will reduce the recurrence rate in carcinoma breast patients.

Keywords: *Lymphatic invasion, blood vessel invasion, Carcinoma breast, D2-40, CD 31, metastasis.*

Introduction

D2-40 is a sensitive and specific marker to detect tumour cells in lymphatic vessels[(1)(2)]. It selectively and specifically stains lymphatic endothelial cells. CD31 is a 130 kDa transmembrane glycoprotein in the immunoglobulin superfamily. CD31 stains blood and lymph vessels[(3)]. D2-40 specifically stains lymph vessels. Identifying lymphovascular invasion (LVI) is easy in many cases but becomes difficult in haematoxylin and eosin stained slides, if lumen is completely filled by tumour cells or when retraction artefacts are present due to tissue shrinkage during fixation. Lymphovascular invasion is associated with high histological grade, high ki67 expression and negative hormone receptor. Metastasis and recurrence are the main cause of mortality in breast carcinoma. Lymphovascular invasion is associated with increased risk of metastasis and recurrence of tumour[(4)]. Tumour cells can reach blood circulation via connection between tumour associated lymphatic and blood circulation. LVI is also associated with distant relapse and decreased survival[(5)]. Therefore tumours with lymphovascular invasion without lymph node involvement may get benefit from adjuvant chemotherapy

Objectives

To assess usefulness of D2-40 and CD 31 in detection of lymphatic and vascular invasion in carcinoma breast compared to routine H&E stain

Procedure

The mastectomy specimens received in the Department of Pathology during a period of 18 months were included in the study. The specimen was fixed in 10% formalin, representative samples were taken and processed. Multiple 3-5 µm sections were taken from advancing edge of tumour and stained with H&E. Lymphovascular invasion was assessed in peritumoural tissue on H&E stained sections. Lymphovascular invasion was identified when carcinoma cells are present within a definite, endothelial lined space. When tumour cells were observed in a space without a definite endothelial lining, it was not considered as emboli. Lymphatics were taken as vascular channels lined by single layer of endothelial cells, occasionaly contaniing lymphocytes, lymphatic fluid and without any RBC'S. Blood vessels were taken as vascular channels with supporting smooth muscle and lumen of which showed RBCs. Sections were also taken for immunohistochemical staining. Immunostaining was performed on sections using routine immunohistochemical procedure. 5 micrometer thick sections were made on poly L Lysine coated slides kept at 37 degree Celsius overnight and at 60 degree Celsius for 1 hour next day followed by antigen retrieval and staining. Antibodies used were D2-40 and CD31[(6)(7)]. Vessels were considered lymphatic when endothelium stained with both D2-40 and CD31. Vessels were classified as blood vessel when endothelium stained only for CD31 and negative for D2-40. Tumour cells seen in D2-40 stained vessels were taken as lymphatic emboli. Tumour cells seen in vessels stained only by CD31 were taken as blood vessel emboli[(8)(9)].

Observation

Data was collected from 60 patients of invasive breast carcinoma and lymphatic and vascular invasion was assessed by H&E and immunohistochemical stains D2-40 and CD 31

By H&E stain Lymphatic invasion was detected in 24 of 60 cases (40%)

By D2-40 invasion was detected in 40 of 60 cases (66.7%).

16 cases of lymphatic invasion which were missed by H&E stain and was detected by D2-40 staining.

In H&E sections Blood vessel invasion was detected in 36 of 60 cases

By CD 31 stain, Blood vessel invasion was detected in 45 of 60 cases

9 cases of vascular invasion which were missed by H&E stain could be detected by CD31 staining.

Thus sensitivity of D2-40 in detecting lymphatic invasion was found to be 83.3%. Specificity was found to be 44.4% and Kappa value was 0.250, p value – 0.049 indicating fair agreement.

Table 1: Sensitivity and specificity of D2-40:

			LVI on H&E		
			Absent	Present	Total
D2-40	LVI absent	Count	16	4	20
		% within D2-40	80.0%	20.0%	100.0%
		% within LVI ON H&E	44.4%	16.7%	33.3%
	present	Count	20	20	40
		% within D2-40	50.0%	50.0%	100.0%
		% within LVI on H&E	55.6%	83.3%	66.7%
Total		Count	36	24	60
		% within D2-240	60.0%	40.0%	100.0%
		% within LVI on H&E	100.0%	100.0%	100.0%

CD 31 showed 100% sensitivity and 62.5% specificity. Kappa for diagnostic agreement (0.667) was substantial, p value less than 0.05

No significant association was observed between lymphatic invasion detected by D2-40 with lymph node metastasis (chi-square value -0.897, p value-0.344)

No significant association between CD31 positive blood vessel invasion and lymph node metastasis (Chi-square value 2.392, p value- 0.122)

No signifcant association was observed between vascular invasion detected by CD31 with tumour size (p value-1)

No signifcant association was observed between lymphatic invasion detected by D2-40 with tumour size (p value-0.439)

No signifcant association was observed between blood vessel invasion detected by H&E with tumour size (p value-0.576)

No signifcant association was observed between lymhatic invasion detected by H&E with age of patient (p value-0.138)

Table 2: sensitivity and specificity of CD31

			BVI on H&E		
			Absent	Present	
CD31 BVI	Absent	Count	15	0	15
		% within CD31	100.0%	.0%	100.0%
		% within BVI on H&E	62.5%	.0%	25.0%
	Present	Count	9	36	45
		% within CD31	20.0%	80.0%	100.0%
		% within BVI on H&E	37.5%	100.0%	75.0%
Total		Count	24	36	60
		% within CD31	40.0%	60.0%	100.0%
		% within BVI on H&	100.0%	100.0%	100.0%

Table 3: Frequency lymph node metastasis in grade 1, grade2, grade3 tumours

	Lymph nodal metastasis	
Grade	Present	Absent
1	0	1
2	26	16
3	11	5
Total	38	22

No statistically signifcant association was observed between lymphatic invasion detected by D2-40 with age of patient (p value-0.271)

No signifcant association was observed between vascular invasion detected by CD31 with age of patient (p value-0.881).

Table 4: Association between lymphatic invasion and grade of tumour

H&E LVI	**Grade**			**Total**
	1	**2**	**3**	
Present	0	17	7	24
Absent	1	26	9	36
Total	1	43	16	60

No statisticantly significant association between lymphatic invasion detected by H&E with grade of tumour (Fishers exact value- 0.865).

No statisticantly significant association between lymphovascular invasion detected by D2-40 with grade of tumour (P=0.480).

No statisticantly significant association between vascular invasion detected by CD 31 with grade of tumour (P=0.188).

Figure: 1 CD 31 stained vessel showing tumour emboli

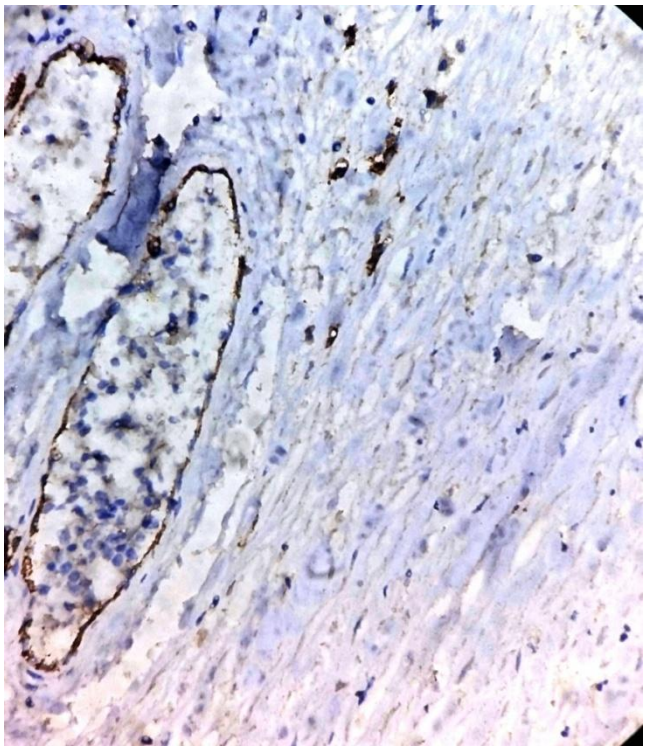
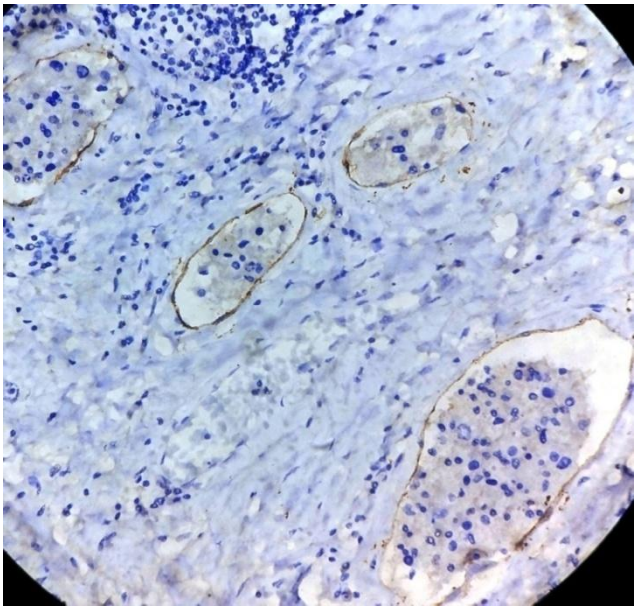


Figure: 2 D2-40 stained slides showing lymphatic emboli



Discussion

In this study 60 cases of invasive breast carcinoma were evaluated. Lymphatic and vascular invasion was assesed on H&E section. Lymphatics were taken as vascular channels lined by single layer of endothelial cells, occasionaly containing lymphocytes, lymphatic fluid and without any

RBC'S. Blood vessels were taken as vascular channels with supporting smooth muscle and lumen of which showed RBC'S.Then sections were stained with D2-40 and CD 31. Tumour cells lying in spaces lined by D2-40 positive cells were taken as lymphatic invasion as it specifically stains lymphatic endothelium only. Tumour cells lying in spaces lined by CD 31 positive cells taken as blood vessel invasion.

Lymphatic invasion was detected by H&E in 24 of 60 cases (40%). Lymphatic invasion was detected by D2-40 staining in 40 of 60 cases (66.7%).Thus increased detection rate of lymphatic invasion was observed with the use of D2-40. Sensitivity of D2-40 in detecting lymphatic invasion was found to be 83.3% and specificity was found to be 44.4%. Kappa for diagnostic agreement was fair (0.250). D2-40 was found to be a useful marker to distinguish between lymphatic invasion and blood vessel invasion According Jung Ah lee et al D2-40 positive lymphatic invasion showed a positive correlation with lymph node metastasis and showed increased incidence of recurrence[10]. Emad A Rakha et al showed a positive association of lymphatic invasion with large tumour size, high tumour grade, high Ki-67 index, negative estrogen receptor expression. So cases with lymphovascular invasion were found to be less amenable to endocrine therapy[11]. According to metaanalytical study conducted by Sandi shen more HER 2 expression noted in tumours with lymphovascular invasion. HER 2 expression found to be associated with poor prognosis and brain metastasis often developed when metastasis occurred[12]. So identifying lymphatic invasion and distinguish it from blood vessel invasion is important.

In this study no significant association was observed between D2-40 lymphatic invasion and metastasis to lymph node (p value- 0.344). However frequency of lymph node metastasis was found to be higher (67.5%) in D2- 40 positive lymphatic invasion group compared to those without D2-40 lymphatic invasion (55%). Smaller sample size might be the reason for not getting a significant association.

According to study conducted by vannesa fortes et al no relationship was observed between tumour size and lymphatic invasion. Lymphatics are draining vessels and not required for tumour growth[13]. In this study no statistically significant association was observed between D2-40 lymphatic invasion with tumour size (p value-0.439).

According to study conducted by Rabaab et al lymphatic invasion more often seen patients less than 50 years[14]. In this study no statistically significant association was observed between D2-40 lymphatic invasion and age of patients (p value-0.271). However frequency of lymphatic invasion was found to be more in 50 or less than 50 years (72.7%) compared to those more than 50 years (59.3%).

According to study by Vaneesa et al lymphatic invasion correlated with high grade possibly due to growth factor production by rapidly proliferating tumour which produces larger variety of clonal tumour cells capable of lymphatic invasion[13]. In this study no statistically significant association was observed between lymphatic invasion and grade of tumour

CD 31 stains both lymph and blood vessel endothelial cells. Blood vessel invasion taken as tumour cells lying within vessels stained by CD31 and not with D2-40. Vaneeza et al found vascular invasion in 4.1% cases in H&E and in 15.4% cases by CD31 staining[13]. Kappa value was 0.198 which showed poor agreement[13]. This study detected vascular invasion by H&E in 36 of 60 cases (60%). CD 31 positive vessels showed invasion in 45 of 60 cases (75%). Thus increased detection rate of blood vessel invasion observed by using CD31 compared to H&E. CD 31 staining had a sensitivity of 100% and specificty of 62.5 % in detection of blood vessel invasion according to this study. kappa value obtained was 0.667. Kappa for diagnostic agreement was substantial for vascular invasion detected by CD31. Thus CD 31 was found to be a useful marker for detection of blood vessel invasion as per our study. According to study by T Kato et al patients with high average microvessel count and blood vessel invasion were associated with increased risk

of cancer related death (15). So identifying blood vessel invasion separately is prognostically important.

According to study by Vanessa fortes et al positive correlation was observed between blood vessel invasion and tumour size more than 2 cm[13]. Out of the 45 cases with blood vessel invasion detected by CD31, 30 cases had tumour size more 2cm according to our study. However no statistically significant association between tumour size and blood vessel invasion was observed (p value- 1) possibly due to small sample size.

According to study by Fadia et al blood vessel invasion was found to be associated with large tumour size and high tumour grade[16]. In this study no statistically significant association was observed between grade and CD 31 positive blood vessel invasion. However frequency of vascular invasion detected by CD 31 was higher (81.2%) in grade 3 tumours compared to grade 2 tumours which showed a frequency of 74.4%. Grade 1 tumour didn't show any lymphovascular invasion.

A study by vanessa et al did not observe any relationship between blood vessel invasion and metastasis[17]. Fadia et al observed positive association between blood vessel invasion and lymphnode metastasis. In this study no signifcant association was observed between vascular invasion detected by CD31 with lymph node metastasis (p value-0.122).

No signifcant association was observed between vascular invasion detected by CD31 with age of patient (p value-0.881). Almost equal frequency of lymphovascular invasion detected in 50 or less than 50 years age group (75.8%) and in more than 50 years age group (74.1%)

38 patients had lymphnode metastasis among 60 cases studied. 22 cases did not have any lymphnode involvement. Among 16 cases with grade 3 tumours 11 had lymph node metastasis. This study showed higher frequency of lymph node involvement in high grade tumours

Lymphatic invasion missed in 16 cases on H&E. Blood vessel invasion missed in 9 cases on H&E. Some of these cases showed vessel lumen completely filled by tumour emboli which became on apparent only on immunohistochemical staining. Some of the cases lacked a definite endothelial cell lining.

No signifcant association was observed between lymhatic invasion detected by H&E with age of patient (p value-0.138).

No signifcant association was observed between blood vessel invasion detected by H&E with tumour size (p value-0.576)

Conclusion

- Increased detection rate of lymphatic invasion was observed with the use of D2-40.
- Sensitivity of D2- 40 in detecting lymphatic invasion was found to be 83.3% and specificity was found to be 44.4%.
- Kappa for diagnostic agreement was fair (0.250)
- D2-40 was found to be a useful marker to distinguish between lymphatic invasion and blood vessel invasion
- Increased detection rate of blood vessel invasion detected by CD31 compared to H&E
- CD 31 staining has a sensitivity of 100% and specificty of 62.5 % in detection of blood vessel invasion according to this study.
- kappa value obtained was 0.667. Kappa for diagnostic agreement was substantial for blood vessel invasion detected by CD31
- CD 31 was found to be a useful marker for detection of blood vessel invasion as per this study
- Frequency of lymph node metastasis was found to be higher (67.5%) in D2- 40 positive lymphatic invasion group compared to those without D2-40 lymphatic invasion (55%).
- Frequency of lymphatic invasion was found to be more in 50 or less than 50 years (72.7%) compared to those more than 50 years (59.3%).
- No signifcant association was observed between vascular invasion and age of patient (p value-0.881)

- no signifcant association was observed between vascular invasion and lymph node metastasis (p value-0.122)
- No signifcant association was observed between lymphatic invasion and lymph node metastasis
- No statisticantly significant association between lymphatic invasion and grade of tumour

References

1. Marinho VF, Sanches FS, Rocha GF, Metze K, Gobbi H. D2-40, a novel lymphatic endothelial marker: identification of lymphovascular invasion and relationship with axillary metastases in breast cancer. Jornal Brasileiro de Patologia e Medicina Laboratorial. 2008 Feb;44(1):45-50.
2. Arnaout-Alkarain A, Kahn HJ, Narod SA, Sun PA, Marks AN. Significance of lymph vessel invasion identified by the endothelial lymphatic marker D2-40 in node negative breast cancer. Modern pathology. 2007 Feb 1;20(2):183-91.
3. Van den Eynden GG, Van der Auwera I, Van Laere SJ, Colpaert CG, van Dam P, Dirix LY, Vermeulen PB, Van Marck EA. Distinguishing blood and lymph vessel invasion in breast cancer: a prospective immunohistochemical study. British journal of cancer. 2006 Jun 5;94(11):1643-9.
4. Britto AV, Schenka AA, Moraes-Schenka NG, Alvarenga M, Shinzato JY, Vassallo J, Ward LS. Immunostaining with D2–40 improves evaluation of lymphovascular invasion, but may not predict sentinel lymph node status in early breast cancer. BMC cancer. 2009 Apr 8;9(1):109.
5. Rakha EA, Martin S, Lee AH, Morgan D, Pharoah PD, Hodi Z, MacMillan D, Ellis IO. The prognostic significance of lymphovascular invasion in invasive breast carcinoma. Cancer. 2012 Aug 1;118(15):3670-80.
6. Dekker TJ, Van de Velde CJ, Van Bruggen D, Mesker WE, Van der Hoeven JJ, Kroep JR, Tollenaar RA, Smit VT. Quantitative assessment of lymph vascular space invasion (LVSI) provides important prognostic information in node-negative breast cancer. Annals of oncology. 2013 Oct 10;24(12):2994-8.19.
7. Wahal SP, Goel MM, Mehrotra R. Lymphatic vessel assessment by podoplanin (D2-40) immunohistochemistry in breast cancer. Journal of cancer research and therapeutics. 2015 Oct 1;11(4):798.
8. Song YJ, Shin SH, Cho JS, Park MH, Yoon JH, Jegal YJ. The role of lymphovascular invasion as a prognostic factor in patients with lymph node-positive operable invasive breast cancer. Journal of breast cancer. 2011 Sep 1;14(3):198-20323.
9. Mohammed ZM, McMillan DC, Edwards J, Mallon E, Doughty JC, Orange C, Going JJ. The relationship between lymphovascular invasion and angiogenesis, hormone receptors, cell proliferation and survival in patients with primary operable invasive ductal breast cancer. BMC clinical pathology. 2013 Nov 25;13(1):31.
10. Tezuka K, Onoda N, Takashima T, Takagaki K, Ishikawa T, Wakasa T, Wakasa K, Hirakawa K. Prognostic significance of lymphovascular invasion diagnosed by lymphatic endothelium immunostaining in breast cancer patients. Oncology reports. 2007 May 1;17(5):997-1003.
11. Lee JA, Bae JW, Woo SU, Kim H, Kim CH. D2-40, podoplanin, and CD31 as a prognostic predictor in invasive ductal carcinomas of the breast. Journal of breast cancer. 2011 Jun 1;14(2):104-11.
12. Shen S, Zhong S, Lu H, Huang W, Xiao G. A Meta-Analysis of Lymphatic Vessel Invasion Correlated with Pathologic Factors in Invasive Breast Cancer. Journal of Cancer Therapy. 2015 Apr 2;6(04):315.
13. Marinho VF, Metze K, Sanches FS, Rocha

GF, Gobbi H. Lymph vascular invasion in invasive mammary carcinomas identified by the endothelial lymphatic marker D2-40 is associated with other indicators of poor prognosis. BMC cancer. 2008 Feb 29;8(1):64.

14. Mohammed RA, Menon S, Martin SG, Green AR, Paish EC, Ellis IO. Prognostic significance of lymphatic invasion in lymph node-positive breast carcinoma: findings from a large case series with long-term follow-up using immunohistochemical endothelial marker. Modern Pathology. 2014 Dec 1;27(12):1568-77.
15. Kato T, Kameoka S, Kimura T, Nishikawa T, Kobayashi M. The combination of angiogenesis and blood vessel invasion as a prognostic indicator in primary breast cancer. British journal of cancer. 2003 Jun 16;88(12):1900-8.
16. Gujam FJ, Going JJ, Mohammed ZM, Orange C, Edwards J, McMillan DC. Immunohistochemical detection improves the prognostic value of lymphatic and blood vessel invasion in primary ductal breast cancer. BMC cancer. 2014 Sep 18;14(1):676.
17. Laser J, Cangiarella J, Singh B, Melamed J, Chiriboga L, Yee H, Darvishian F. Invasive lobular carcinoma of the breast: role of endothelial lymphatic marker D2-40. Annals of Clinical & Laboratory Science. 2008 Mar 20;38(2):99-104.