Original Article

DOUBLE ANTIBODY COCKTAIL (D2-40/P63) PROTOCOL FOR IDENTIFICATION OF LYMPHATIC VASCULAR INVASION IN BREAST TISSUES: ADVANTAGES OF THE PROTOCOL

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Summary

Recently it was found out that D2-40/p63 double immunostaining of defined lymph vessel invasion has additional prognostic value in some node-negative breast carcinomas. The aim of this study was to test the efficiency of our modification of D2-40/ p63 double antibody cocktail, used with single detection kit protocol to identify lymphatic vascular invasion (LVI) in breast tissues. Formalin-fixed, paraffin-embedded tissues from 130 breast cancer patients were examined. Immunostaining for α-SMA, collagen IV, p63 and D2-40 was performed in accordance with protocols provided by the manufacturer (DAKO). An additional immunostaining with D2-40/p63 was performed according to a protocol modified in our laboratory. Using D2-40 staining only, two cases of extensive lobular cancerization by ductal carcinoma in-situ (DCIS) and two cases of solid low-grade DCIS were misdiagnosed as lymphatic vascular invasion. In cases with in-situ carcinomas /extensive intraductal and/or lobular tumor growth, the D2-40 / p63 cocktail proved useful in identifying true LVI. The strength of inter-method agreement between evaluation of LVI on D2-40-stained slides and D2-40 / p63 stained slides in breast carcinomas was very good (κ=0.90). The suggested single detection kit, double antibody cocktail (D2-40/ p63) staining protocol enabled effective interpretation of the nature of tumour cell harbouring D2-40 positive structures in all the cases we studied.

Key words: D2-40/p63, breast cancer, ductal/ lobular carcinoma in-situ, lymphatic vascular invasion

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"He still remembered the Morse code.
All he had to do in order to break through
To people in the outside world was to
Lie in bed and dot dash to the nurse."

Johnny Got His Gun
Dalton Trumbo

Introduction

Lymphatic vascular invasion (LVI) is recognized to be a predictor of survival in breast cancer [1], but its accurate evaluation is not always possible on hematoxilin and eosin (H&E) stained specimen. The anipodoplanin antibody clone D2-40 was found to

be a highly selective marker of lymphatic endothelium [2]. It has also been reported that D2-40 staining for LVI identification enables more reliable prediction of lymph node metastases as compared to the H&E-stained sections [3]. In breast tissues, D2-40 was found to be expressed by myoepithelial cells (MEC). This has raised concerns about the risk of overestimation of LVI due to positivity in MEC surrounding ducts and/or lobuli harbouring carcinomatous growth, and therefore the use of additional myoepithelial staining was suggested [4].

Recently it was found out that D2-40/p63 double immunostaining defined lymph vessel invasion has additional prognostic value on some node negative breast carcinomas [5]. Nearly simultaneously, we introduced our preliminary data on D2-40/p63 double immunostaining [6].

The aim of the study was to test the efficiency of our modification of D2-40/ p63 double antibody cocktail, a single detection kit protocol for identification of lymphatic vascular invasion in breast tissues.

Materials and Methods

Materials/Patients

Formalin-fixed (10% NFB), paraffin-embedded tissues from 130 breast cancer patients were examined (mean age 62.36 years, age range 30-82). Details concerning the patients studied are presented on Table 1.

Special attention was paid to cases in which there were MEC surrounded structures (ducts and/or lobuli harbouring carcinomatous growth). The presence of in-situ carcinoma (ductal and/or lobular) as well as lobular cancerization (partial involvement by ductal carcinoma in-situ cells of a group of lobules within a terminal duct lobular unit) and pagetoid spread of lobular carcinoma in-situ filling and expanding ducts (involvement of extra lobular ducts by lobular carcinoma in-situ) in the evaluated for LVI tissue blocks was assessed. The nature of the intraductal and/or intralobular malignant proliferation was interpreted in accordance with morphological and immunohistochemical criteria applied at the time of initial histopathological diagnosis [described in details in 7-10].

Immunohistochemical method

Five consecutive 4m thick tissue samples were prepared from one selected tissue block from

Table 1. Patient population

Tumor size (p T)	Axillary node status (pN)	(Tumor grade) G	LVI evaluate on D2 - 40/p63 stained
p T1 – 39 (30.00%) p T2 – 36 (27.69%) p T3 - 4 (3.08%) p T4 – 10 (7.69%) p T x – 41 (31. 54%)	pN (– ve) – 49 (37.70%) pN(+ ve) – 60 (46.15%) pN (x) - 21 (16.15%)	G 1 – 21 (16.15%) G 2 – 75 (57.69%) G 3 – 14 (10.77%) Gx – 20 (15.38%)	LVI – ve 87 (66.92%) LVI +ve 24 (18.46%) LVI - x 19 (14.62%)

Histological types of breast carcinoma according to WHO [7]	
Invasive ductal carcinoma	106 (81.54%)
Invasive lobular carcinoma	7 (5.38%)
Mucinous carcinoma	4 (3.08%)
Cribr iform carcinoma	1 (0.77%)
Papillary carcinoma	1 (0.77%)
Neuroendocrine carcinoma	1 (0.77%)
Mixed type (has features of both invasive ductal and lobular carcinoma)	7 (5.38%)
Undiffer e ntiated	1 (0.77%)
Medullary	2 (1.54%)

x – not certain / unknown; – ve – negative; +ve – positive

each case. Histological materials were deparaffinised in xylene, and rehydrated using ethanol in a series of decreasing concentrations. Immunostaining for α-SMA, collagen IV, p63 and D2-40 was performed and interpreted in accordance with protocols provided by the manufacturer DAKO.

Details on the used modified D2-40/p63 immunohistochemical staining protocol are presented on Table 2.

Table 2. Details on the main steps of the IHC staining procedure with D2-40/p63

	Protocol properties	**D2-40/p63 protocol single detection kit protocol**
Pre-analytic phase	**Method used**	En vision
	Main buffer	TRIS/EDTA; pH=7.2-7.4
	Automation	NONE
	Endogenous Block	yes
	Peroxide	H_2O_2 0.3% in methanol;
	Incubation	
	Chromogen & Substrate	
	Chromogen	DAB/Tris Buffer
	Working conc.	0.01mg/ml
	Substrate	H_2O_2 /Tris Buffer pH=7.4; Temp:24°C; Time 5 min
	Antigen retrieval	
	Heat mediated	pressure cooking press16 pa t=124 C
	Buffer	TRIS/EDTA vol: 0.25L Molarity 10; pH= 9 Time 1 min
	Antibody	
	Primary Antibody	I. D2-40 (Clone: D2-40 Isotype: IgG1, kappa
	Clone / supplier	Mouse IgG) /DAKO
	Dilution	1:100
		+(added as a cocktail)
		II
	Time	p63 (Clone: 4A4 (1). Isotype: IgG2a, kappa.
	Temperature	Mouse IgG) / DAKO
		1/25
	Detection kit	
	Clone / supplier	EnVision HRP/DAB / DAKO
	Dilution	Prediluted
	Time	
	Temperature	30 °C 30 min
	Counter stain	Mayer hematoxylin
Analyticphase	Criteria for interpretation of the staining used:	lymphatic vessels: "continuous" intermediate to strong cytoplasm staining (lymphatic vessel reactivity for D2-40), myoepithelial cells: "bead –like" staining (weak to missing patchy cytoplasm D2-40 staining and strong p63 nuclear staining).

Statistical analysis

Choen's Kappa analysis was used to determine the strength of inter-method agreement between D2-40 alone and D2-40 / p63 [11]. The specificity and sensitivity of D2-40 and D2-40/p63 staining for LVI evaluation was calculated (p63, α-SMA and collagen IV staining were used as additional markers, when discrepancy between results from D2-40 staining and D2-40/p63 staining occurred).

Results

In normal breast tissues D2-40 staining did not cause any interpretation concerns (Figure 1).

Nineteen (14.62%) of the studied cases were considered as uninterpretable for LVI, since no D2-40 positive structures (neither lymphatic vessels nor ductolobular units were observed) - the histological specimens were entirely composed of tumour cells and fibrosis.

In 42 (32.31%) cases, tissue blocks evaluated for LVI contained either in in-situ tumours, or extensive intraductal and/or intralobular tumour spread Table 3.

In some cases, LVI was indistinguishable from extensive intraductal and/or lobular tumour growth on both H&E and D2-40 stained slides. In these cases, interpretation of α-SMA and collagen IV staining could be also equivocal and therefore not particularly useful (Figure 2).

In cases with in-situ carcinomas (extensive intraductal and/or lobular tumor growth) the D2-40 / p63 cocktail proved useful for identification

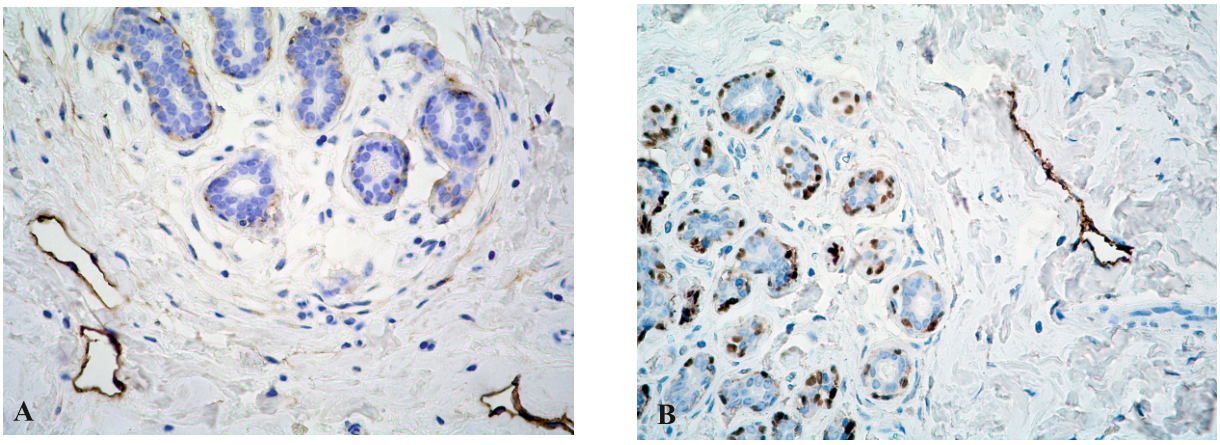

Figure 1. A) D2-40 staining - weak patchy cytoplasmic staining of myoepithelium of terminal ducts and lobules and strong continuous staining of lymphatic vessels 400x, D2-40; B) D2-40 / p63 staining - strong nuclear p63 positivity combined with weak (pale) patchy cytoplasmic staining of myoepithelium of terminal ducts and lobules and strong continuous staining of lymphatic vessels. 400x, D2-40 / p63

Table 3. Incidence of presence of LVI coexisting with in situ tumours.
Decision was made on D2-40 and consecutive p63 α-SMA and collagen IV stained slides and data from the initial histomorphological assessment, retrieved from archival documentation

	DCIS/DIN	LCIS / LIN	Extensive ductal growth of lobular carcinoma	Extensive lobular growth (cancerization by ductal carcinoma in-situ)
LVI absent	28	3	1	4
Both LVI and intraductal / lobular growth	5	0	0	1

All results were in accordance with the D2-40 / p63 cocktail stained slides interpretation. DCIS/ DIN – ductal carcinoma in-situ/ ductal intraepithelial neoplasia; lobular carcinoma in-situ / lobular intraepithelial neoplasia

Table 4. Intermethod agreement between evaluation of LVI on D2-40 and D2-40/p63

n= 111 (19 uninterpretable cases are excluded)	D2-40/p63 negative	D2-40/p63 positive
D2-40 negative	83	0
D2-40 positive	4	24

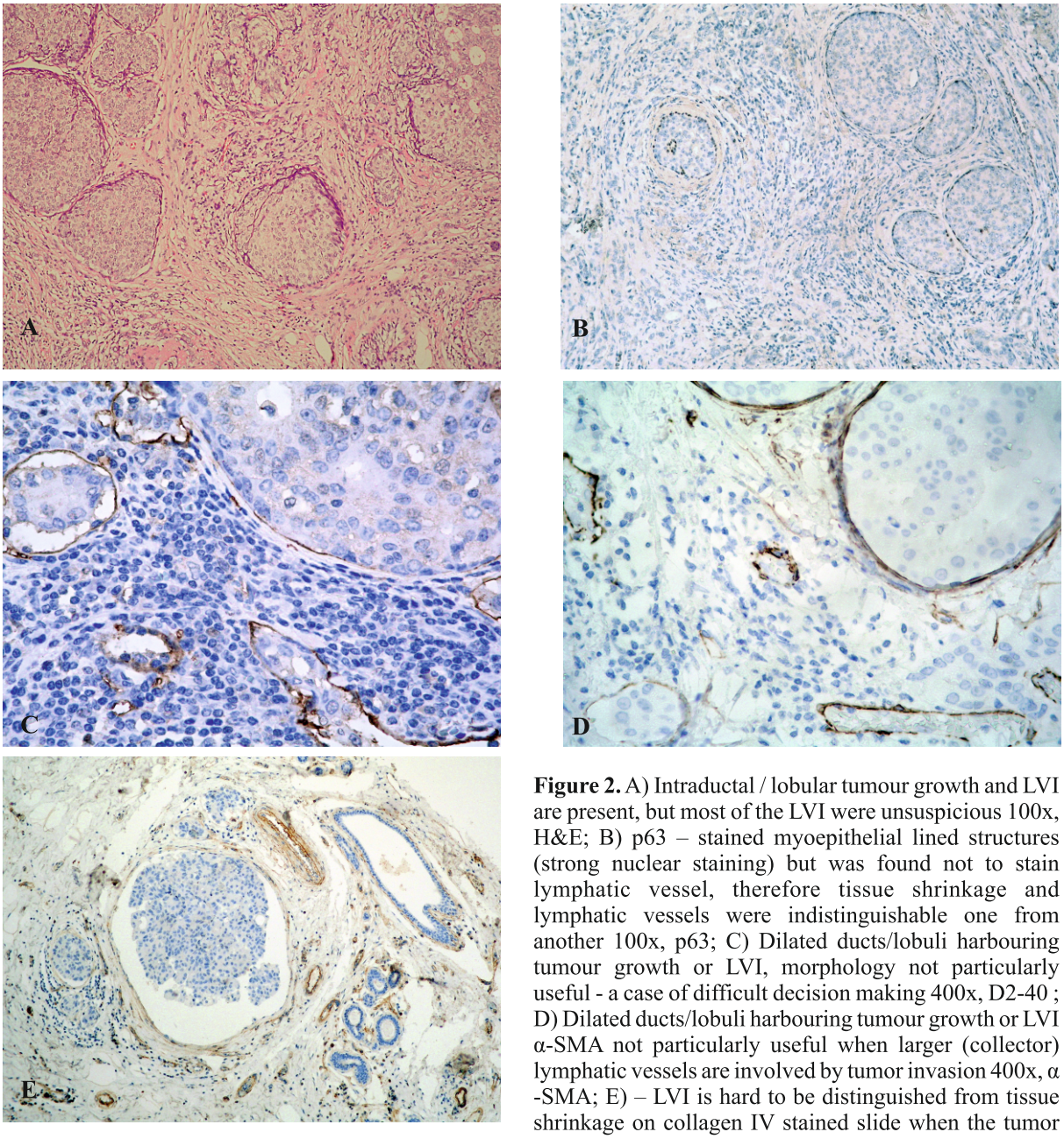

Figure 2. A) Intraductal / lobular tumour growth and LVI are present, but most of the LVI were unsuspicious 100x, H&E; B) p63 – stained myoepithelial lined structures (strong nuclear staining) but was found not to stain lymphatic vessel, therefore tissue shrinkage and lymphatic vessels were indistinguishable one from another 100x, p63; C) Dilated ducts/lobuli harbouring tumour growth or LVI, morphology not particularly useful - a case of difficult decision making 400x, D2-40 ; D) Dilated ducts/lobuli harbouring tumour growth or LVI α-SMA not particularly useful when larger (collector) lymphatic vessels are involved by tumor invasion 400x, α -SMA; E) – LVI is hard to be distinguished from tissue shrinkage on collagen IV stained slide when the tumor cells are situated in lymphatic capillaries. One huge cluster in a precollector lymphatic vessel with positive basement membrane and adjacent smaller calibre lymphatic vessel with only partial basement membrane staining 100x, Collagen IV.

of true LVI (Figure 3).

The strength of inter-method agreement between evaluation of LVI on D2-40 stained slides and D2-40 / p63 stained slides in breast carcinomas was very good κ=0.90 (Table 4).

Two cases of extensive lobular cancerization by ductal carcinoma in-situ (DCIS) and two cases of solid, low grade DCIS were erroneously diagnosed as LVI. Sensitivity and specificity for LVI detection in breast cancer, detected with D2-40 antibody alone was 100% and 95.40%, respectively, when interpreted in the context of staining with p63, α-SMA and collagen IV. D2-40/p63 was found to have virtually 100% sensitivity and 100% specificity for LVI detection, when interpreted in the context of p63, α-SMA and collagen IV.

In one of the four cases discussed (65 year-old, with invasive ductal carcinoma NOS with extensive in-situ component pT1c, Nx 0/6; Mx; G2 LVI +ve, true negative; ER moderately positive in over 60%; HER2 – 1+), the application

of D2-40/p63 protocol and D2-40 alone might have resulted in changes of the treatment plan.

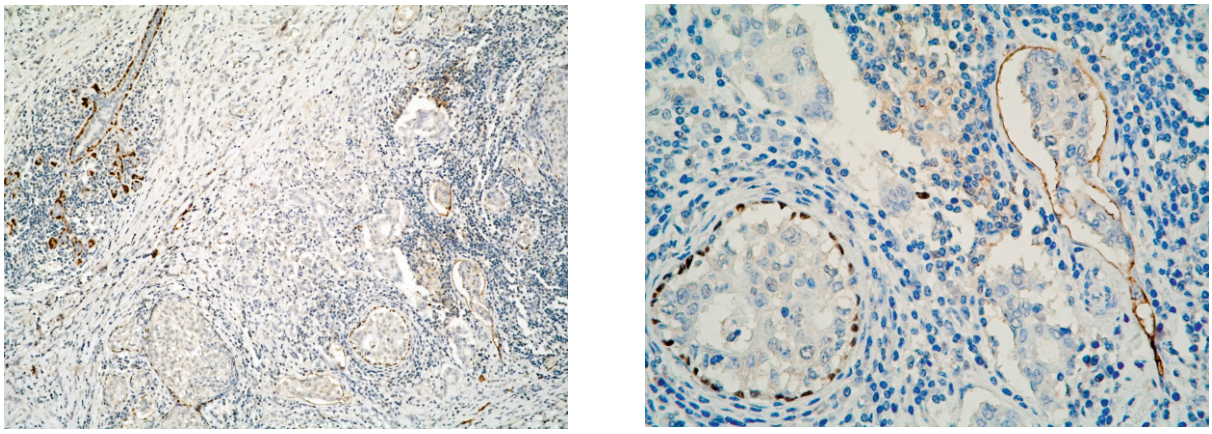

Figure 3. A) D2-40/ p63 cocktail stained materials, lymphatic vessels demonstrated "continuous" intermediate to strong cytoplasm staining (lymphatic endothelial cell reactivity for D2-40), readily distinguishable from the "bead-like" staining observed in MEC of distorted duct lobular units (weak to missing cytoplasm D2-40 staining, strong p63 nuclear staining). 100x, D2-40/ p63; B)High magnification showing intraductal tumour growth – "bead-ike" staining, LVI – "continuous" intermediate to cytoplasm staining and tissue shrinkage around tumour nests – no immunoreactivity 400x, D2-40/ p63.

Discussion

Protocols of the two primary antibodies (D2-40 and p63) required similar techniques and conditions, thus allowing their combined use on a single slide. Furthermore, their distinct staining patterns - cytoplasmic D2-40 and nuclear p63 positivity, allowed for histological interpretation even with the application of a single detection kit.

Intraductal and/or intralobular tumour growth is likely to be found in about 1/3 of the tissue specimens evaluated for LVI. According to data in literature, the rate of in-situ carcinomas combined with invasive carcinoma may reach 75.8% [12]. It means that decision-making on LVI in breast cancer might be difficult even more often than reported here. Additionally stained slides with α-SMA and collagen IV were not particularly useful in most of the cases. The difficulties encountered when interpreting histological findings in α-SMA- stained slides were found to be associated with the presence of smooth muscles in the wall of pre-collector and collector lymphatic vessels, which were indistinguishable from the flattened myoepithelium of dilated ductolobular units. In contrast to interpreting findings in α-SMA-stained slides, collagen IV interpretation was difficult in lymphatic capillaries, which were with incomplete or even missing basal membrane. Tumor emboli in such vessels were indistinguishable from artificial tissue shrinkage around tumor nests on collagen IV stained tissue slides.

The suggested single detection kit, double antibody cocktail (D2-40/ p63) staining protocol enabled effective interpretation of the nature of tumour cell harbouring D2-40 positive structures in all studied cases, that contained positive D2-40 structures.

Despite the concerns raised about considerable interpretation difficulties of lymphatic vascular invasion arising from D2-40 staining of myoepithelial cells [4], the results from D2-40 (D2-40 alone) staining in this study demonstrated specificity and sensitivity over 95%. Only in foci of intraductal and/or intralobular tumour growth, when the ducts/lobuli were distorted and myoepithelial cells were flattened, the intraductal/lobular tumour growth could be misinterpreted as LVI.

Some tissue shrinkage can also cause interpretation difficulties on H&E staining. In such cases, IHC staining with D2-40/ p63, D2-40 alone or α-SMA would be sufficient for making a reliable diagnosis.

Because of the wide scope of criteria set for identifying LV I: all D2-40 positive cell-lined structures, containing tumour cells and an endothelium appearing as a crisp, wire-like pattern, that completely outlines the entire circumference of the lymphatic space [4], no LVI was erroneously recognized as intraductal and/or intralobular tumour growth on D2-40 alone

stained slides.

If the suggested D2-40/p63 protocol is used instead of D2-40 alone, this could influence the choice of treatment in at least 0.90% of patients from a random population.

We support the idea of Gudlaugsson E at al. for recognition of D2-40/p63 protocol as a prognostic factor in breast cancer [5].

Conclusion

The application of double antibody cocktail (D2-40/ p63) protocol for identification of lymphatic vascular invasion in breast tissues is reliable and useful method for identification of LVI in cases with accompanying in-situ tumor.

References

1. Goldhirsch A, Ingle JN, Gelber RD, Coates AS, Thurlimann B, Senn HJ, Panel members Thresholds for therapies: highlights of the St Gallen International Expert Consensus on the Primary Therapyof Early Breast Cancer 2009. Ann Oncol. 2009;20(8):1319-29.
2. Kahn HJ, Bailey D, Marks A. Monoclonal antibody D2–40, a new marker of lymphatic endothelium, reacts with Kaposi's sarcoma and a subset of angiosarcomas. Mod Pathol. 2002;15(4):434-40.
3. Marinho VFZ, Metze K, Sanches FSF, Rocha GFS, 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;8:64. doi:10.1186/1471-2407-8-64.
4. Rabban JT, Chen YY. D2-40 expression by breast myoepithelium: potential pitfalls in distinguishing intralymphatic carcinoma from in situ carcinoma. Hum Pathol. 2008;39(2):175-83.
5. Gudlaugsson E, Skaland I, Undersrud E, Janssen EAM, Søiland H. Baak JPA. D2-40/p63 defined lymph vessel invasion has additional prognostic value in highly proliferating operable node negative breast cancer patients Modern Pathol. 2011;24: 502-11.
6. Ivanov I, Popovska S, Betova T, Dineva T. Single detection kit, double antibody cocktail (D2-40/ p63) protocol for identification of lymphatic vascular invasion in breast tissues containing extensive intraductal/-lobular tumour growth. Virchows Arch. 2011;459(Suppl 1):S1-S329.
7. Tavassoli FA, Devillee P. WHO Classification of Tumours: Pathology and Genetics of Tumours of the Breast and Female Genital Organs. Lyon: IARC Press; 2003. p. 11-58.
8. Tavassoli FA. Ductal intraepithelial neoplasia: risk factors for subsequent development of invasive carcinoma. In: Tavassoli FA, editor. Pathology of the breast. Stanford: Appleton & Lange; 1999. p. 205-312.
9. Vicini FA, Goldstein NS, Kestin LL. Pathologic and technical considerations in the treatment of ductal carcinoma in situ of the breast with lumpectomy and radiation therapy. Ann Oncol. 1999;10:883-90.
10. Chen YY, Rabban J T Patterns of Lobular Carcinoma In Situ and Their Diagnostic Mimics in Core Needle BiopsiesPathology. Case Reviews. 2009;14:141-6.
11. Cohen J. Weighted kappa: Nominal scale agreement provision for scaled disagreement or partial credit. By Psychological Bulletin. 1968;70(4):213-20.
12. Tot T. Distribution of the lesions in 500 consecutive breast cancer cases documented on large format histological sections. Cancer. 2007;110:2551-60.