Immunohistochemical expression of D2-40, VEGF and PCNA as biological markers of lymphangiogenesis, angiogenesis and proliferation in pleomorphic adenoma of salivary gland origin

Hiba Jasim Rassol, B.D.S. [(1)]
Kadhim Al-Soudani, B.D.S, M.Sc. [(2)]

ABSTRACT

Background: Pleomorphic adenoma is the most common benign salivary gland tumor and shows a pronounced morphological complexity and diversity; for this The immunoprofiles and clinical course of PA differed according to cellular differentiation. Therefore, it is important to assess potential biomarkers in diagnostic and therapeutic trials. This study evaluates the immunohistochemical expression of D2-40, VEGF and PCNA as markers of lymphangiogenesis, angiogenesis and proliferation of PA and their correlation with clinicpathological parameters and with each other.

Materials and Methods: Twenty five formalin – fixed, paraffin – embedded tissue blocks were included in this study. After histopathological reassessment of haematoxylin & eosin stained sections for each block, an immunohistochemical staining was performed using anti D2-40, anti VEGF and anti PCNA monoclonal antibodies.

Results: Positive immunohistochemical of D2-40, VEGF and PCNA was found in (100%), (92%) and (88%) of the cases respectively. No statistically significant correlation neither among the markers immunoexpression nor with the clinicopathological finding, except a statistical significant correlation was found between (D2-40andVEGF) expression with the histopathological presentation of the cases.

Conclusion: D2-40, VEGF and PCNA immunoexpression showed significant correlation with respect to the histopathological presentation of the cases. While no significant correlation seen regarding the expression of aforementioned markers with each other, suggests that each marker might affect on tumor behavior independently.

Keywords: Pleomorphic adenoma, D2-40, VEGF, PCNA. (J Bagh Coll Dentistry 2013; 25(Special Issue 1):53-58).

INTRODUCTION

Pleomorphic adenoma (PA) is a common benign salivary gland neoplasm characterized by neoplastic proliferation of parenchymatous glandular cells along with myoepithelial components [(1)]. Blood and lymphatic vascular systems are very important for supplying cells with nutrients and oxygen and for removing excessive fluids, which are crucial tasks for hemodynamic maintenance; so is an important issue for cell nutrition not only in malignant but also in benign tumor [(2-4)]. D2-40 is a novel monoclonal antibody to O-linked sialoglycoprotein that reacts with affixation – resistant epitope, 40 kD a sialoglycoprotein found on lymphatic endothelium, glandular myoepthelial cells, fetal testis and on the surface of testicular germ cell tumors [(5)]. Vascular endothelial growth factor (VEGF) is a signal protein produced by cells that stimulates vasculogenesis and angiogenesis.

It is part of the system that restores the oxygen supply to tissues when blood circulation is inadequate active in angiogenesis, vasculogenesis and endothelial cell growth. Induces endothelial cell proliferation, promotes cell migration, inhibits apoptosis [(6,7)].

The expression of genes related to cell proliferation and oncogenesis seems to be associated with the prognosis of the PA. Tissue growth depends on both the rate of cell proliferation and cell death. The increased ability of cells in PA to proliferate is suggesting a tendency of tumor toward recurrence and possible susceptibility of PA to malignant transformation [(8)]. Proliferating Cell Nuclear Antigen, commonly known as PCNA, is a protein that acts as a processivity factor for DNA polymerase δ in eukaryotic cells. It achieves this processivity by encircling the DNA, thus creating a topological link to the genome. It is an example of a DNA clamp. In response to DNA damage, this protein is ubiquitinated and is involved in the RAD6-dependent DNA repair pathway [(9)]. The biological knowledge of PA plays a crucial role in their diagnosis, treatment and prognosis .The evaluation of the expression of different proteins can reflect facts about the biology and behavior of this tumor.

This study aimed to asses:

- Immunohistochemical expression of (D2-40), (VEGF) and (PCNA) as biological markers of lymphangiogenesis, angiogenesis and proliferation in pleomorphic adenoma of salivary glands.

(1) Master student, Department of Oral Diagnosis, College of Dentistry, University of Baghdad.
(2)Assistant Professor, Department of Oral Diagnosis, College of Dentistry, University of Baghdad.

- Correlation of studied markers with clinicopathological finding of pleomorphic adenoma and with each other.

MATERIALS AND METHODS:

Twenty five formalin – fixed, paraffin – embedded tissue blocks were collected from labrotaries archives and included in this study. After histopathological reassessment of haematoxylin & eosin stained sections for each block. Four micrometer thick sections were cut and mounted on positively charged slides and stained immunohistochemically with monoclonal antibodies using anti D2-40, anti VEGF and anti PCNA monoclonal antibodies (Abcam UK). Abcam expose mouse and rabbit HRP/DAB immunohistochemical detection kit (Catalog No. ab80436, Cambridge, UK) was used.

RESULTS

The sample comprised 14 (56%) females and 11 (44%) males with a female/male ratio (1.27:1). The age range of the patients with pleomorphic adenoma was between (16 -59) years with a mean of (36.5600 ±12.51026). The majority of the cases 20 (80%) was located in parotid gland followed by palate 5 (20%) cases. Histopathological classifying was performed for each case of pleomorphic adenoma as follows, cellular and classic types were represented in 10 (40%) of the studied cases for each, while stromal type was presented in 5 (20%) of the cases (Table 1).

D2-40 immunostaining revealed positive lymphatic vessels immunoreactivity in intratumoral lymphatic vessel density (ILVD) was recorded in 7 cases (28%) out of 25 with a mean of (0.7600±1.755942) whereas lymphatic endothelial cells that limited to peritumoral lymphatic vessel density (PLVD) was recorded in 4 cases (16%) out of 25 with a mean of (1.0000±2.107131). (Table 2 and figure1&2).

Myoepithelial cells showed D2-40 +v immunostaining in all studied cases (Table 3 and figure 3&4).

Regarding immunohistochemical expression of VEGF and PCNA monoclonal antibodies, positive immunostaining was seen in 23 cases (92%) and 22 cases (88%) respectively (Table 4 and figure5) (Table 5 and figure 6)

There was no significant statistical correlation neither among the markers immunoexpression nor with the clinicopathological finding, except a statistical significant correlation was found between (D2-40 and VEGF) expression with the histopathological presentation of the cases (Table 6&7).

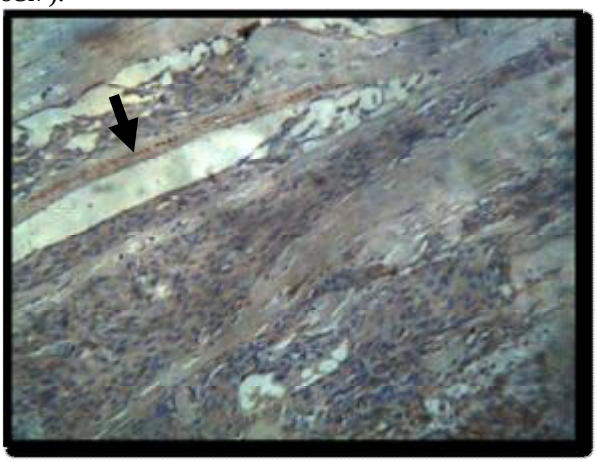

Figure 1: Photomicrograph shows D2-40 immunostaining -positive lymphatic vessels in PA (arrow). (Original magnification X 200)

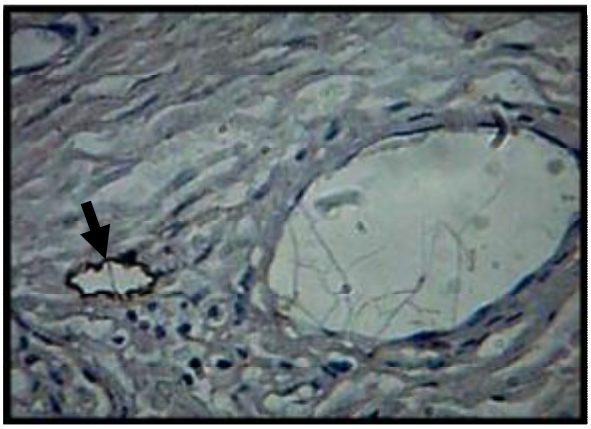

Figure 2: Photomicrograph shows D2-40 immunostaining-positive lymphatic vessel (arrow).The adjacent blood vessel is negative (Original magnification X400)

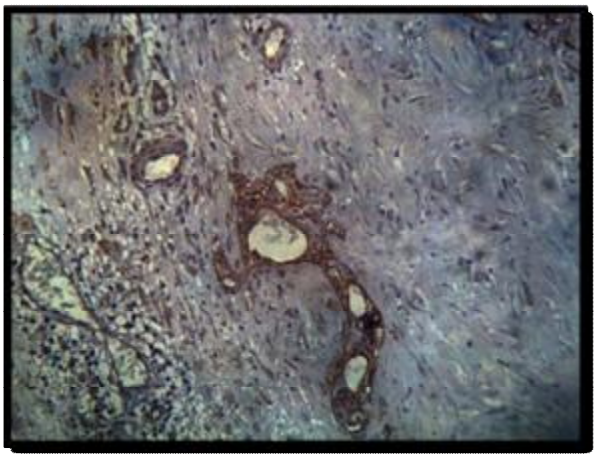

Figure 3: Photomicrograph shows positive D2-40 immunostaining in myoepithelial cells (PA). (Original magnification X200)

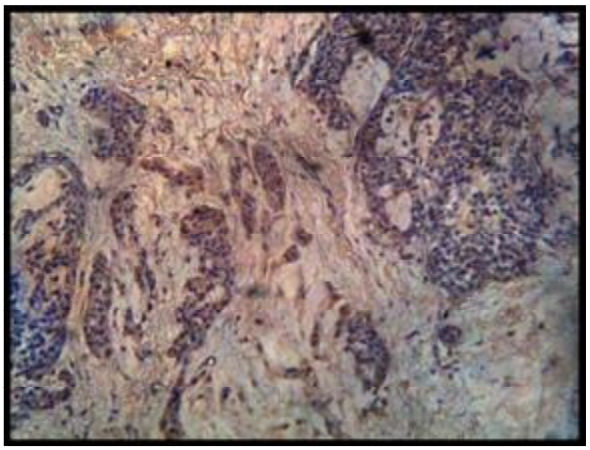

Figure 4: Photomicrograph showing positive D2-40 immunostaining in myoepithelial cells (PA). (Original magnification X200)

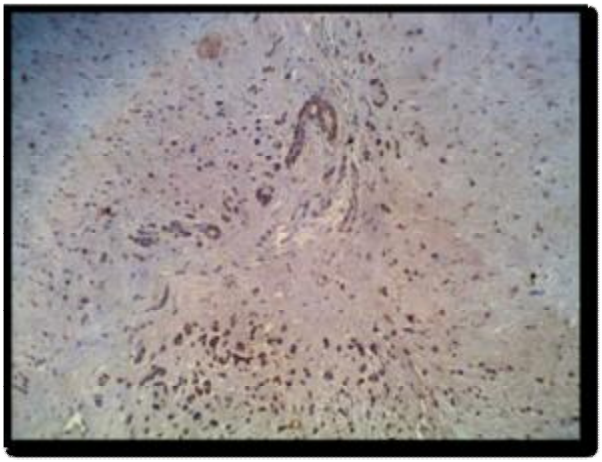

Figure 5: Photomicrograph shows positive VEGF immunostaining in duct like structure, myoepithelial cells and stromal cells of PA.(Original magnification X100)

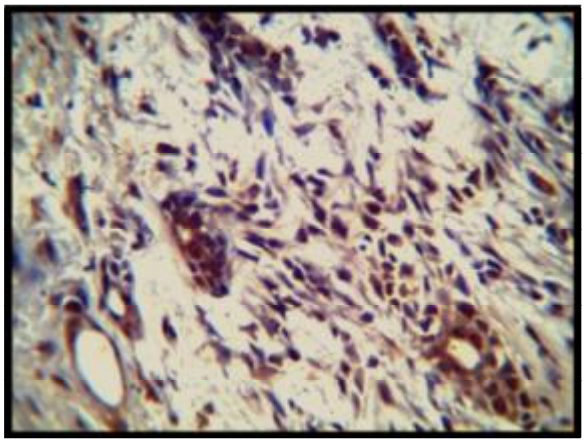

Figure 6: Photomicrograph shows positive nuclear PCNA immunostaining in epithelial/myoepithelial cells of PA. (Original magnification X400)

DISCUSSION

Concerning the epidemiological parameters, including age, sex, site, clinical presentation, studies showed variable results; These inconsistent findings among different studies could be credit with the fact that the current study and some of the others are not an epidemiological type of studies, therefore the limited number and the random selection of the cases according to what is available preclude for definitive clinical findings.

Assessment of D2-40 immunohistochemical expression:

A) Assessment of Lymphatic Vessel Density (LVD):

In this study D2-40 immunostaining revealed positive lymphatic vessels immunoreactivity in intratumoral lymphatic vessel density (ILVD) was recorded in 7 cases (28%) out of 25 with a mean of (0.7600±1.755942) whereas lymphatic endothelial cells that limited to peritumoral lymphatic vessel density (PLVD) was recorded in 4 cases (16%) out of 25 with a mean of (1.0000±2.107131).

B) Assessment of D2-40 Expression in Tumor Cells.

a) Assessment of D2-40 Expression in Myoepithelial Cells:

As it was expected, in this study when D2-40 was applied to demonstrate lymphatic vessels in PA cases, the myoepithelial cells showed positive expression to it in all the studied cases in the basal cells of glandular structures as well as in stellate/spindle cells in myxochondroid matrices. This is in agreement with many researches [9,12-15].

b) Assessment of D2-40 Expression in Epithelial Cells:

Regarding epithelial cells, D2-40 immunostaining was not detected in this study. Similar findings found by *Soares et al,(2007)* who reported that neoplastic epithelial cells showed enhanced membranous positivity in carcinoma ex-PA but not in PA without malignant transformation. From the above findings it is possible that no normal epithelial cells expressed D2-40 and to assume that D2-40 act as a good tumor marker in the differential diagnosis of certain carcinomas from their potential histologic mimics since the presence of morphological similarities between the cells of some neoplastic lesions and their normal or benign counterparts impose diagnostic difficulties [16, 12,17,18].

Regarding clinicopathological variables; the present study showed statistically non significant correlation were identified except a statistical significant correlation was found between D2-40 immunoexpression with histopathological presentation of the cases; however, no previous reports highlights this correlation.

Assessment of VEGF Expression:

This study showed a positive expression to VEGF antibody in 23 (92%) of the cases. This finding comes in accordance with the results of previous study performed by Pammer et al. [19] who demonstrated VEGF mRNA and protein in (100%) of PA cases. The majority of the cases showed weak (32%) to moderate (48%) immunoreactivity, this could be confined to metabolically low rate of the tumor that maintain metabolic activity via an oxygen-independent process [20].

As far as correlation of VEGF expression with clinicopathological parameters of PA is concerned; the present study showed statistically non significant correlation were identified except a statistical significant correlation was found between VEGF immunoexpression with histopathological presentation of the cases; however, no previous reports highlights this correlation.

Assessment of PCNA Expression:

The current study, IHC results of 25 cases of PA revealed positive PCNA expression in 22 (88%) of the cases, this findings is similar to other findings [21,22] who found positive PCNA expressed in (78.9%) and (75%) of PA cases respectively.

The current results for most cases showed a predominantly weak PCNA labeling 10 (40%) or no labeling 3 (12%). This result agrees with the those of [23-26] who reported a predominance of weak expression of this protein in PA, could be attributed to the less aggressive behavior of PA and possibly may indicate a lower tendency of the studied cases toward recurrence and possible susceptibility of these lesions to malignant transformation.

Regarding clinicopathological variables; the present study showed statistically no significant difference neither among different age nor between males and females. Similarly non-significant correlation was found between PCNA expression and different tumor site, this is in accordance with the findings of two researches [27,28].

As far as the histolpathological aspect is concerned, the present study showed non-significant correlation in PCNA expression scores among different tumor histopathological subtypes**.**

The correlation between the biological markers in PA:

Among all the available studies reviewed, to the best of our knowledge, the present work is the first to study these biomarkers all together except several studies that assessed only one of the aforementioned markers [19,5,11,12].

There was non-significant statistical correlation neither among the markers immunoexpression nor with the clinicopathological finding, except a statistical significant correlation was found between (D2-40 and VEGF) expression with histopathological presentation of the cases. This study reveals that of the aforementioned markers were positivity expression by most of the studied cases suggests that each of them may act independently of each other in influencing of the tumor behavior.

REFERENCES

1. Eveson JW, Kusafuka K, Stenman G, et al. In: Barnes L, Eveson JW, Reichart P, Sidransky D, editors. Pathology and Genetics of Head and Neck Tumors. Lyon: IARC Press; 2007: 254- 8.
2. Reis-Filho JS, Schmitt FC. Lymphangiogenesis in tumors: what do we know? Microsc Res Tech 2003; 60(2): 171–80.
3. Choi WW, Lewis MM, Lawson D, et al. Angiogenic and lymphangiogenic microvessel density in breast carcinoma: correlation with clinicopathologic parameters and VEGF family gene expression. Mod Pathol 2005; 18: 143–52.
4. Maby-El Hajjami H, Petrova TV. Developmental and pathological lymphangiogenesis: From models to human disease. Histochem Cell Biol 2008; 130(6):1063-78.
5. Al-Rawi NH, Omar H, Al- Kawas S. Immunohistochemical analysis of P_{53} and bcl-2 in benign and malignant salivary glands tumors. J Oral Pathol Med 2010; 39:48-55. **(IVSL).**

6. Monsky WL, Fukumura D, Gohongi T, Ancukiewcz M, Weich HA, Torchilin VP, Yuan F, and Jain RK. Augmentation of transvascular transport of macromolecules and nanoparticles in tumors using vascular endothelial growth factor. Cancer Res 1999; 59: 4129-4135.
7. Mesri M, Morales-Ruiz M, Ackermann EJ, et al. Suppression of vascular endothelial growth factor-mediated endothelial cell protection by surviving targeting. Am J Pathol 2001; 158(5):1757-*65*.
8. Alves FA, Pires FR, de Almeida OP, et al. PCNA, Ki-67 and p53 expressions in submandibular salivary gland tumors. Int J Oral Maxillofac Surg 2004; 10: 593- 97.
9. Leonardi E, Girlando S, Serio G, et al."PCNA and Ki67 expression in breast carcinoma: Correlations with clinical and biological variables". J. Clin. Pathol. 1992; 45 (5): 416– 20.
10. Teymoortash A, Schrader C, Shimoda H, et al. Evidence of lymphangiogenesis in Warthin's tumor of the parotid gland. Oral Oncol 2007; 43(6): 614- 8.
11. Soares AB, Juliano PB, Araujo VC, Metze K, Altemani A. Angiogenic switch during tumor progression of carcinoma ex-pleomorphic adenoma. Virchows Arch 2007; 451(1):65-71.
12. Soares AB, Ponchio L, Juliano PB, et al. Lymphatic vascular density and lymphangiogenesis during tumor progression of carcinoma ex pleomorphic adenoma. J Clin Pathol 2007; 60(9): 995–1000.
13. De Araujo VC, Altemani A, Furuse C, et al. Immunoprofile of reactive salivary myoepithelial cells in intraductal areas of carcinoma ex-pleomorphic adenoma. Oral Oncol 2006; 42:1011-6.
14. Tsuneki M, Satoshi Maruyama, Manabu Yamazaki, et al. Podoplanin is a novel myoepithelial cell marker in pleomorphic adenoma and other salivary gland tumors with myoepithelial differentiation. Virchows Archiv 2012: 0945- 55.
15. Sarkis SA, Younis WH, Al-Ani S. Salivary gland tumors in Iraqi children and adolescents. J Bagh Coll Dentistry 2010; 22(3):74-6.
16. Ren S, Abuel-Haija M, Khurana JS, et al. D2-40: An additional marker for myoepithelial cells of breast and the precaution in interpreting tumor lymphovascular invasion, Int J Clin Exp Pathol 2011; 4(2):175- 82.
17. Wang D, Li Y, He H, et al. Intraoral minor salivary gland tumors in a Chinese population: A retrospective study on 737 cases. Oral Surg. Oral Med. Oral Pathol Oral Radiol Endod 2007; 104: 94-100.
18. Browning L, Bailey D, Parker A. D2-40 is a sensitive and specific marker in differentiating primary adrenal cortical tumors from both metastatic clear cell renal cell carcinoma and phaeochromocytoma. J Clin Pathol 2008; 61:293- 96.
19. Pammer J, Weninger W, Mildner M, et al. Vascular endothelial growth factor is constitutively expressed in normal human salivary glands and is secreted in the saliva of healthy individuals. J Pathol 1998; 186(2):186-91.
20. Costa AF, Demasi APD, Bonfitto VLL, et al. Angiogenesis in salivary carcinomas with and without myoepithelial differentiation. Virchows Arch 2008; 453*:* 359–67.
21. Gorden-Bnenz MA, Godoy GP, Soares RC, et al. Imunohistochemical expression of PCNA, p53 and bcl-2 in pleomorphic adenomas. Int J Morphol 2008; 26(3): 567- 72.
22. Rivera-Batidas H, Ocanto RA, Azevedo AM. Intraoral minor salivary gland tumours: A retrospective study of 62 cases in Venezuelan population. J Oral Pathol Med 1996; 25:1-4.
23. Yang L, Hashimura K, Qin CH, Shrestha ZP, et al. Immunoreactivity of proliferating cell nuclear antigen in salivary gland tumors: An assessment of growth potential. A Pathol Anat 1993; 86: 422: 81.
24. Matturri L, Lavezzi AM, Biondo B, Mantovani M. Cell kinetics of pleomorphic adenomas of the parotid gland. Eur J Cancer B Oral Oncol 1996; 32B:154– 57.
25. Trendell-Smith NJ, Oates J, Crocker J. The evaluation of salivary gland tumors using proliferating cell nuclear antigen. J Laryngo Otol 1997; 111: 551- 5.
26. Zhu Q, White F H, Tipe G L. The assessment of proliferating cell nuclear antigen (PCNA) immunostaining in human benign and malignant epithelial lesions of the parotid gland. Oral Oncol 1997; 33:29-35.
27. Daniele E, Tralongo V, Morello V, et al. Pleomorphic adenoma and adenoid-cystic carcinoma of salivary glands: Immunohistochemical assessment of proliferative activity in comparison with flow-cytometric study. Cell Prolif 1996; 29(3): 153- 62.
28. Da Cruz Perez DE, De Abreu Alves F, et al. Prognostic Factors In Head And Neck Adenoid Cystic Carcinoma. Oral Oncol 2006; 42(2):139-146.

Table 1: Clinicopathological characteristic of 25 PA cases

Age	No.	%
15-24	5	20
25-34	5	20
35-44	9	36
45-54	2	8
Sex		
Male	11	44
Female	14	56
Tumor site		
Parotid	20	80
Palate	5	20
Histological type		
Cellular	10	40
Stromal	5	20
Classic	10	40

Table 2: Labeling index of the ILVD, PLVD, TLVD in 25 cases of PA

	N	Min.	Max.	Mean	S.D.
ILVD	25	0.00	6.00	0.7500	1.755942
PLVD	25	0.00	8.00	1.0000	2.107131
TLVD	25	0.00	12.00	1.8000	3.329164

Table 3: D2-40 expression scores in 25 cases of PA

D2-40 expression	No. of cases	%
Score 1	13	52
Score 2	5	20
Score 3	7	28

Table 4: VEGF expression scores in 25 cases of PA

VEGF expression	No. of cases	%
Score 0	2	8
Score1	8	32
Score2	12	48
Score3	3	12

Table 5: PCNA expression scores in 25 cases of PA:

PCNA expression	No. of cases	%
Score 0	3	12
Score1	10	40
Score2	10	40
Score3	2	8

Table 6: Correlation between D2-40 and tumor histopathological subtypes in PA

D2-40 scores	Histopathological subtypes						Total		X	Sig.
	Cellular type of PA		Stromal type of PA		Classic type of PA		N	%		
Score1	1	10.0%	5	100%	7	70.0%	13	52.0	8.231	0.01
Score2	2	20.0%	0	0.0%	3	30.0%	5	20.0		
Score3	7	70.0%	0	0.0%	0	0.0%	7	28.0		
Total	10	100.0%	5	100%	10	100.0%	25	100 %		

Table 7: Correlation between VEGF and tumor sites in PA

VEGF scores	Histopathogical subtypes						Total		X	Sig.
	Cellular type of PA		Stromal type of PA		Classic type of PA		N	%		
Score0	0	0.0%	0	0.0%	2	20.0%	2	8.0	16.354	0. 012
Score1	2	20.0%	5	100%	1	10.0%	8	32.0		
Score2	6	60.0%	0	0.0%	6	60.0%	12	48.0		
Score3	2	20.0%	0	0.0%	1	10.0%	3	12.0		
Total	10	100.0%	5	100%	10	100.0%	25	100.0		