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Epidermal growth factor receptor (EGFR) overexpression in triple-negative breast cancer: association with clinicopathologic features and prognostic parameters

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Abstract

Introduction: Triple-negative breast cancers are a poor prognostic group of breast cancers that don't respond to conventional hormonal and her2neu targeted therapy. A subset of triple-negative breast cancer is known to overexpress epidermal growth factor receptor (EGFR); however prognostic significance of this biomarker has not been widely studied in our population. Therefore, we aimed to evaluate the frequency of EGFR overexpression in triple-negative breast cancer in our setup and its association with prognostic and predictive factors.

Methods: We performed EGFR immunohistochemistry on 150 cases of triple-negative breast cancers. Intensity and percentage of EGFR expression were combined to formulate an EGFR score, that was compared with prognostic features of breast cancer and recurrence status of patients.

Results: Positive EGFR expression was noted in 18.7% (28 cases); out of which 16% (24 cases) showed low EGFR expression, whereas high EGFR expression was seen in 2.7% (4 cases). No significant association of EGFR expression was noted when compared with various clinicopathological parameters and recurrence status of the patients.

Conclusion: We found EGFR protein expression in 18.7% of cases while high expression was seen in only 2.7 % cases of triple-negative breast cancer which may harbor underlying genetic alterations like altered EGFR gene copy number, chromosome 7 copy number or average EGFR gene: chromosome 7 ratio; therefore we suggest that molecular tests like FISH to evaluate these EGFR molecular alterations should be performed in EGFR over expressing triple negative breast cancers in our setup to identify patients that can benefit from anti-EGFR targeted therapy. Moreover, regional difference in EGFR expression (high expression in chinese population compared to our population) are may be due to different underlying genetic alterations in triple-negative breast cancers, further necessitating a need of devising personalized therapeutic protocols for locoregional population.

Keywords: Triple-negative breast cancers, EGFR, CK5/6, epidermal growth factor

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Introduction

Triple-negative Breast Cancers (TNBC) are a poor prognostic group of breast cancers and accounts for 20% of human breast cancers (Swain, 2008; Nakai et al., 2016; Kim et al., 2006; Hashmi et al., 2014a; Hashmi et al., 2014b; Hashmi et al., 2018a). TNBC are diagnosed pathologically by < 1% expression of Estrogen Receptor (ER) and Progesterone Receptor (PR) and Human Epidermal Receptor 2 (Her2neu) 0/1+ or 2+ (FISH negative) as per CAP/ ASCO recommendation (Hammond et al., 2010; Wolff et al., 2013). Due to lack of expression of hormonal and Her2neu receptors; conventional targeted therapies (hormonal and anti-her2neu) are ineffective in TNBC. Human Epidermal Growth Factor Receptor (EGFR) consists of several transmembrane glycoproteins. Activating mutations of EGFR results in uncontrolled cell proliferation and survival (Masuda et al., 2012). Such mutations of EGFR have been reported in many human cancers especially lung adenocarcinoma, head and neck squamous cell carcinoma and colorectal carcinoma (Scagliotti et al., 2004; Chung et al., 2005; Hashmi et al., 2018b; Hashmi et al., 2018c). Therefore EGFR represents a useful therapeutic target for EGFR inhibitor therapy. At present anti-EGFR therapy has not been approved in the treatment of TNBC and therefore routine EGFR testing is not recommended. Moreover, there are no set guidelines for the immunohistochemical interpretation of EGFR overexpression. It has been noted that EGFR abnormalities exhibit geographic and ethnic variations and its expression varies in different regions of the world (Nakajima et al., 2014; Grob et al., 2012; Secq et al., 2014; Tilch et al., 2014; Jacot et al., 2011).EGFR overexpression has not been evaluated in breast cancers of our population. Therefore in this study we aimed to evaluate the EGFR overexpression in TNBC in our population and its prognostic significance.

Methods

Total 150 cases of TNBC were retrieved from records of pathology department. The surgical specimens were those of modified radical mastectomies, simple mastectomies and wide local excisions with sentinel lymph node dissection. All of these patients had surgeries at Liaquat National hospital, Karachi from January 2008 till December 2013. Hematoxylin and eosin stained slides of all of these cases were reviewed independently by two senior histopathologists and pathologic characteristics were recorded. Clinical records of all patients were also retrieved and reviewed from institutional files to evaluated patients age and recurrence status. Moreover, representative blocks of each case were selected for immunohistochemistry. ER, PR and Her2neu immunohistochemistry were done to reconfirm triple-negative status. CK5/6 immunohistochemistry was done to subcategorize TNBC cases into basal and non-basal subtypes.

Immunohistochemistry

ER, PR, Her2neu and Ki67 IHC were performed using DAKO antibodies as shown below, with EnVision^{TM} FLEX, high pH DAKO kit according to manufacturer's protocol.

1. FLEX Monoclonal Rabbit Anti-human Estrogen Receptor alpha, Clone EP1.
2. FLEX Monoclonal Mouse Anti-human Progesterone receptor clone PgR 636
3. Polyclonal Rabbit Anti-human c-erbB-2 oncoprotein
4. FLEX Monoclonal mouse Anti-human Ki67 Antigen clone MIB-1

For ER and PR, nuclear staining in >1% of cancer cells was taken as positive. For her2neu, cases were scored 1 +, 2+ and 3+ according to CAP guidelines. For equivocal cases (2+) fluorescent in situ hybridization (FISH) was done using Path Vysion Her2neu probe kit according to manufacturer's recommendations. Paraffin embedded tissues were cut at 5 microns and mounted on positively charged glass slides. Four slides were prepared, with one slide stained with hematoxylin and eosin. Well preserved invasive carcinoma was marked on the H & E slide as the target area. Target area was etched with a diamond tipped scriber on the back of the unstained slide to be assayed. Glass slides with paraffin embedded tissue sections were baked at 56 degree centigrade overnight. Tissue was then processed in xylene, ethanol, purified water and wash buffer. Probe mixture was applied to the target area of the tissue on slide, sealed with rubber cement and then incubated in thermobrite for hybridization. After post-hybridization buffering, DAPI (counter stain) was applied. Slides were interpreted by two pathologists, evaluating 60 interphase nuclei using fluorescence microscope equipped with appropriate excitation and emission filters allowing the visualization of the orange and green fluorescent signals (Hashmi et al., 2014b). Results were expressed as ratio of her2neu signals as compared to CEP17 signals according to ASCO/CAP guidelines (Wolff et al., 2014).

For Ki67 immunostaining, proportion of positive stained cancer cells were recorded (Jalava et al., 2006). Furthermore on the basis of Ki67 index, cases were categorized into <15%, 16-24%, 25-44% and >44% ki67 index categories.

CK5/6 IHC was performed by using FLEX Monoclonal Mouse Anti-human Cytokeratin 5/6, clone D5/16 B4 by using DAKO envision kit. Intermediate to strong cytoplasmic and membranous staining in more than 10% cells was considered positive CK5/6 expression.

EGFR immunohistochemistry was performed using DAKO EnVision method using DAKO Monoclonal Mouse Anti-human Epidermal growth factor Receptor (EGFR), clone H11 according to manufacturers protocol.

IHC staining was performed manually in batches of 10-15 slides. Positive and negative controls were run along with each batch. For IHC staining, 3-4 micrometer sections were cut on DAKO IHC coated slides. After fixation of slides in oven at 70-80 degree centigrade for 20 mins, sections were de-waxed in xylene and then processed through decreasing concentrations of alcohol after which antigen retrieval was performed. For antigen retrieval, Slides were placed in antigen retrieval solution in water bath at 99-100 degree centigrade for 40 mins. After being kept at room temperature, slides were washed with wash buffer solution 2-3 times, followed by blocking peroxidase for 10 mins in humidity chamber. Subsequently, slides were washed 2-3 times with washing buffer. Primary antibody (EGFR) was applied with dilution of 1:200 for 20 mins, then washed 2-3 times. After application of secondary antibody, slides were incubated for 20mins and then again washed with wash buffer, followed by application of DAB chromogen solution (Wen et al., 2013). Both membranous and cytoplasmic staining for EGFR were evaluated. Intensity of staining was categorized into no staining (0), weak (1+), intermediate (2+), strong (3+) as shown in Fig. 1 as follows

Weak intensity 1+: Faint, incomplete staining of membranes and focal weak positivity in cytoplasm which is only evident on 100X magnification.

Moderate intensity 2+: Complete and moderate staining of membranes and weak to moderate staining in cytoplasm easily appreciable on 40X magnification.

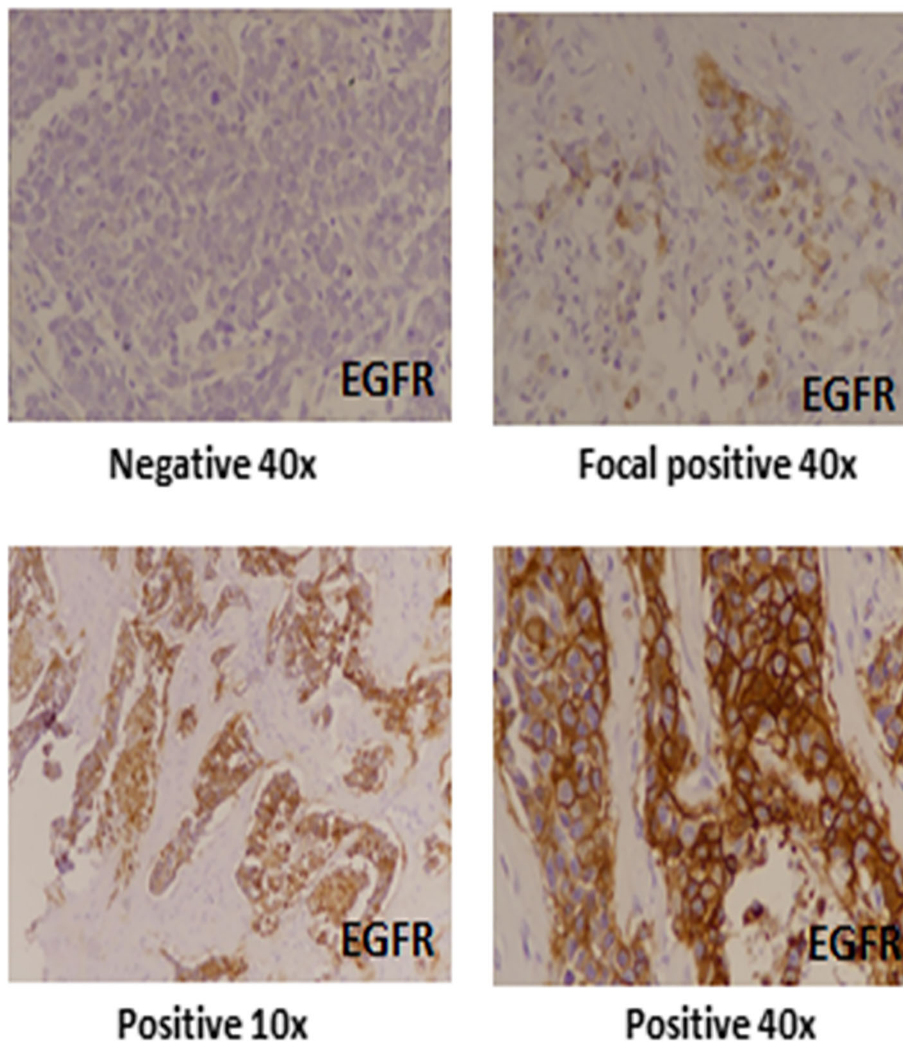

Fig. 1 EGFR expression in triple negative breast cancer. Membranous and cytoplasmic staining was considered positive

Strong intensity 3+: Complete and strong staining of membranes and moderate to strong staining of cytoplasm appreciable on 40X magnification.

Percentage of positively stained cells was also measured and scored as a continuous variable. Intensity score and percentage scored were multiplied to formulate an H-score (0-300). Cases with EGFR expression were further categorized into low and high EGFR expression with a cut-off H-score of 200.

Follow-up and recurrence

Recurrence status and follow-up were evaluated by reviewing patient's hospital record. Overall survival was defined as the time from surgical excision till death or last follow-up. Disease free survival was considered as the time between surgical excision and local recurrence or distant metastasis, last follow-up or patient's death.

Statistical Analysis

Statistical package for social sciences (SPSS 21) was used for data compilation and analysis. Mean and standard deviation were calculated for quantitative variables. For qualitative variables, frequency and percentage were calculated. Chi-square test was performed to determine the association. Student t test or Mann witney test were applied to compare difference in means among groups. Survival curves were plotted using Kaplan- Meier method and the significance of difference between survival curves were determined using log-rank ratio. *P*-value ≤0.05 was taken as significant.

Results

Mean age of the patients was 48.9 years. Median follow up time was 28.4+8.3 months. Most common presentation was at stage T2 (52.7%). Most tumors were high grade (grade III), 86.7%. Similarly, 53.35 of cases showed

Table 1 Association of EFGR expression with clinicopathologic features of triple negative breast cancer

		n(%)				*P*-Value
		No Expression (*n*=122)	Low Expression (*n*=24)	High Expression (*n*=4)	Total (*n*=150)	
Age Group	≤30 years	5 (4.1)	0 (0)	0 (0)	5 (3.3)	0.264
	31-50 years	69 (56.6)	11 (45.8)	4 (100)	84 (56)	
	>50 years	48 (39.3)	13 (54.2)	0 (0)	61 (40.7)	
Ki67 Index	≤15%	15 (12.3)	2 (8.3)	0 (0)	17 (11.3)	0.646
	15-24%	6 (4.9)	2 (8.3)	0 (0)	8 (5.3)	
	25-44%	35 (28.7)	7 (29.2)	3 (75)	45 (30)	
	>44%	66 (54.1)	13 (54.2)	1 (25)	80 (53.3)	
Tumor Size/T stage	T1(≤2 cm)	22 (18)	4 (16.7)	0 (0)	26 (17.3)	0.954
	T2(2.1-4 cm)	64 (52.5)	13 (54.2)	2 (50)	79 (52.7)	
	T3(>4 cm)	36 (29.5)	7 (29.2)	2 (50)	45 (30)	
Nodal Stage	N0	74 (60.7)	12 (50)	2 (50)	88 (58.7)	0.539
	N1	24 (19.7)	4 (16.7)	2 (50)	30 (20)	
	N2	10 (8.2)	3 (12.5)	0 (0)	13 (8.7)	
	N3	14 (11.5)	5 (20.8)	0 (0)	19 (12.7)	
Tumor grade	Grade I	1 (0.8)	0 (0)	0 (0)	1 (0.7)	1.000
	Grade II	16 (13.1)	3 (12.5)	0 (0)	19 (12.7)	
	Grade III	105 (86.1)	21 (87.5)	4 (100)	130 (86.7)	
Histological subtypes	IDC	106 (86.9)	18 (75)	3 (75)	127 (84.7)	0.353
	Papillary	4 (3.3)	2 (8.3)	0 (0)	6 (4)	
	Medullary	1 (0.8)	0 (0)	0 (0)	1 (0.7)	
	Metaplastic	9 (7.4)	4 (16.7)	1 (25)	14 (9.3)	
	Mixed	2 (1.6)	0 (0)	0 (0)	2 (1.3)	
Lymphovascular Invasion	Present	27 (22.1)	8 (33.3)	1 (25)	36 (24)	0.470
	Absent	95 (77.9)	16 (66.7)	3 (75)	114 (76)	
Triple negative phenotypes	Basal	10 (8.2)	6 (25)	0 (0)	16 (10.7)	0.064
	Non Basal	112 (91.8)	18 (75)	4 (100)	134 (89.3)	

Fisher exact test was applied
P-value≤0.05, considered as significant

high ki67 index (>44%). Nodal metastasis was seen in 42.7% cases. Although 84% cases had invasive ductal carcinoma, NST; a significant proportion of cases were of metaplastic histology (9.3%). Lymphovascular invasion was present in 24% cases, while 10.7% cases were of basal phenotype based on CK5/6 expression as shown in Table 1.

Positive EGFR expression was noted in 18.7% (28 cases); out of which 16% (24 cases) showed low EGFR expression, whereas high EGFR expression was seen in 2.7% (4 cases). No significant association of EGFR expression was noted when compared with various clinicopathological parameters of TNBC (Table 1). Similarly no significant association of EGFR expression was noted with recurrence status of the patients (Fig. 2). Association of clinicopathologic parameters of TNBC with EGFR was also evaluated using different H-score cutoffs, however no significant association was seen except for histologic subtype as shown in Table 2.

Discussion

In the present study, we found that a small subset of TNBC of our population was found to have EGFR overexpression by immunohistochemistry (IHC) while no significant association was found between EGFR over expression and various prognostic parameter of TNBC.

EGFR overexpression in TNBC markedly varies in different populations. A study conducted in chinese population revealed as high as 42% EGFR expression in TNBC cases (Wang et al., 2015). On other hand, in another study consisting of 567 TNBC cases in Korean population revealed; 1+ EGFR expression in 11.8 % , 2+ in 8.5 % & 3+ in only 3.2 % cases of TNBC & except for lymphovascular invasion , no significant association was found between EGFR expression & various prognostic parameters in TNBC (Kim et al., 2017). These findings were concordant with the results of our study. Similarly

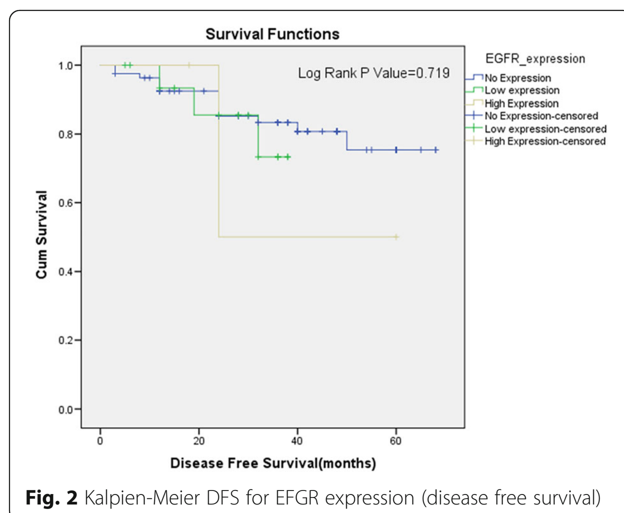

Fig. 2 Kalpien-Meier DFS for EFGR expression (disease free survival)

Park et. al, reported EGFR over expression associated with tumor stage , while association with other prognostic parameter was not significant (Park et al., 2014a). In the current study we used H-score incorporationg both the intensity and percentage of cells to evaluate EGFR expression. In most of the previous only positive EGFR expression was correlated with clinicopathologic variables without categorizing EGFR expression into low or high expression. In most of these studies only intensity of EGFR expression was taken into account and categorized into weak, moderete and strong expression neglecting the percentage of positivity stained cells (Changavi et al., 2015). Due to heterogenity of tumor cells, there is usually variable expression of IHC markers and therfere its always good to score IHC expression, but unfortunately as no standard protocol of interpreting EGFR IHC expression is there, therefore we used different cutoffs.

One of the limitations of our study was that mutation analysis of EGFR gene was not performed as positive over expression does not necessarily correlates with underlying gene mutation in TNBC; and it is necessary to document gene amplification or activating mutations of EGFR in order to benefit from anti-EGFR targeted therapy. Moreover, mechanism of protein over expression in the absence of activating gene mutations is not well understood. Overall EGFR gene mutation is relatively low in TNBC. Studies failed to reveal such mutations in European , Australian & Japanese patients, while 3.4% and 1-2% of cases showed EGFR gene mutations in a subset of American & Korean populations respectively (Reis-Filho et al., 2006; Santarpia et al., 2012; Kim et al., 2013). A frequency of 10% & 11.4% EGFR mutations were reported in Chinese population (Lv et al., 2011a). These findings reveal marked geographic differences in EGFR amplifying gene mutations which may result in protein overexpression in TNBC . High IHC Expression of EGFR may be due to various underlying genetic alterations involving EGFR gene or chromosome 7, including EGFR gene amplification and chromosomal alterations like disomy, low trisomy, high trisomy, low polysomy and high polysomy (Lee et al., 2015).

Conclusion

We found EGFR protein expression in 18.7% of cases while high expression was seen in only 2.7 % cases of triple negative breast cancer which may harbor underlying genetic alterations like altered EGFR gene copy number, chromosome 7 copy number or average EGFR gene: chromosome 7 ratio; therefore we suggest that molecular tests like FISH to evaluate these EGFR molecular alterations should be performed in EGFR over expressing triple-negative breast cancers in our set up to

Table 2 Association of EFGR expression with clinicopathologic features of triple negative breast cancer using different EGFR score cutoffs

		n(%)						*P*-Value
		<50 (*n*=139)	50-100 (*n*=5)	101-150 (*n*=1)	151-200 (*n*=1)	>200(*n*=4)	Total (*n*=150)	
Age Group	≤30 years	5 (3.6)	0 (0)	0 (0)	0 (0)	0 (0)	5 (3.3)	0.207**
	31-50 years	78 (56.1)	1 (20)	1 (100)	0 (0)	4 (100)	84 (56)	
	>50 years	56 (40.3)	4 (80)	0 (00	1 (100)	0 (0)	61 (40.7)	
Ki67 Index	≤15%	17 (12.2)	0 (0)	0 (0)	0 (0)	0 (0)	17 (11.3)	0.298**
	15-24%	7 (5)	1 (20)	0 (0)	0 (0)	0 (0)	8 (5.3)	
	25-44%	41 (29.5)	0 (0)	0 (0)	1 (100)	3 (75)	45 (30)	
	>44%	74 (53.2)	4 (80)	1 (100)	0 (0)	1 (25)	80 (53.3)	
Tumor Size	T1(≤2 cm)	26 (18.7)	0 (0)	0 (0)	0 (0)	0 (0)	26 (17.3)	0.805**
	T2(2.1-4 cm)	73 (52.5)	2 (40)	1 (100)	1 (100)	2 (50)	79 (52.7)	
	T3(>4 cm)	40 (28.8)	3 (60)	0 (0)	0 (0)	2 (50)	45 (30)	
Nodal Stage	N0	82 (59)	3 (60)	0 (0)	1 (100)	2 (50)	88 (58.7)	0.536**
	N1	27 (19.4)	1 (20)	0 (0)	0 (0)	2 (50)	30 (20)	
	N2	12 (8.6)	1 (20)	0 (0)	0 (0)	0 (0)	13 (8.7)	
	N3	18 (12.9)	0 (0)	1 (100)	0 (0)	0 (0)	19 (12.7)	
Tumor grade	Grade I	1 (0.7)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0.7)	0.435**
	Grade II	17 (12.2)	2 (40)	0 (0)	0 (0)	0 (0)	19 (12.7)	
	Grade III	121 (87.1)	3 (60)	1 (100)	1 (100)	4 (100)	130 (86.7)	
Histological subtypes	IDC	120 (86.3)	4 (80)	0 (0)	0 (0)	3 (75)	127 (84.7)	0.034*
	Papillary	4 (2.9)	1 (20)	0 (0)	1 (100)	0 (0)	6 (4)	
	Medullary	1 (0.7)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0.7)	
	Metaplastic	12 (8.6)	0 (0)	1 (100)	0 (0)	1 (25)	14 (9.3)	
	Mixed	2 (1.4)	0 (0)	0 (0)	0 (0)	0 (0)	2 (1.3)	
Lymphovascular Invasion	Present	33 (23.7)	1 (20)	1 (100)	0 (0)	1 (25)	36 (24)	0.560**
	Absent	106 (76.3)	4 (80)	0 (0)	1 (100)	3 (75)	114 (76)	
Triple negative phenotypes	Basal	14 (10.1)	2 (40)	0 (0)	0 (0)	0 (0)	16 (10.7)	0.318**
	Non Basal	125 (89.9)	3 (60)	1 (100)	1 (100)	4 (100)	134 (89.3)	

Fisher exact test was applied
P-value≤0.05, considered as significant

identify patients that can benefits from anti-EGFR targeted therapy. Moreover, regional difference in EGFR expression (high expression in chinese population compared to our population) are may be due to different underlying genetic alterations in triple-negative breast cancers, further necessitating a need of devising personalized therapeutic protocols for locoregional population.

Abbreviations
ER: Estrogen receptor; IHC: Immunohistochemistry; PR: Progesterone receptor; TNBC: Triple negative breast cancer

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Please contact author, Atif Ali Hashmi (doc_atif2005@yahoo.com) for data

Authors' contributions
AAH and SN: main author of manuscript, have made substantial contributions to conception and design of study. SKH, MI, ZFH, EYK, HA and NF: have been involved in requisition of data. SKH, MI, ZFH, EYK, HA and NF have been involved in analysis of the data and revision of the manuscript. All authors read, revise and gave approval of the manuscript.

Ethics approval and consent to participate
Ethics committee of Liaquat National Hospital, Karachi, Pakistan approved the study. Written informed consent was obtained from the patients for the participation.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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