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ORIGINAL ARTICLE

Expression of the RAC1, RHOA and CXCR4 proteins and their interaction as risk factors for infiltration to the nipple areola complex in operable breast carcinoma

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Abstract

Background Nipple areola complex (NAC) infiltration in operable breast carcinoma (OBC) is associated with local recurrence. NAC infiltration in OBC suggests that RAC1, RHOA and CXCR4 proteins are risk factors for migration and infiltration of OBC to NAC. This study aims to analyze the expression and interactions of these proteins as risk factors for NAC infiltration in OBC.

Materials and methods This is an analytic observational cross-sectional study coupled with a categorical comparative study in each 40 subjects of OBC with and without NAC infiltration. The immunohistochemistry performed with a cut-off point based on the result of a receiver operating characteristics (ROC).

Results RAC1, $p < 0.001$ with POR 5.76, 95% CI: 2.06–16.08; RHOA, $p < 0.001$ with POR 7.00, 95% CI: 2.28–21.53; and CXCR4, $p = 0.001$ with POR 6.33, 95% CI 2.06–19.49. There was an interaction between RAC1 and RHOA ($p < 0.001$ with POR 17.14, 95% CI: 3.07-125.66); between RAC1 and CXCR4 ($p < 0.001$ with POR 30.93, 95% CI 3.62–686.89); between RHOA and CXCR4 ($p < 0.001$ with POR 10.21, 95% CI 2.19–54.17); and between the RAC1, RHOA and CXCR4 proteins ($p < 0.001$ with POR = 23.69, 95% CI 2.51–544.86).

Conclusion We conclude that the expression of the RAC1, RHOA, and CXCR4 proteins and their interactions play a role as risk factors of NAC infiltration.

Keywords Operable breast cancer · RAC1 · RHOA · CXCR4 protein · NAC infiltration

Background

Breast cancer (BC) is a malignant neoplasm arising from the epithelial cell duct, the breast node lobule region and the terminal duct known as the terminal duct lobular unit (TDLU) [1]. BC is the most common cancer in females worldwide, with an incidence ranging from 30 cases per 100,000 in East Africa to 137.3 cases per 100,000 in the United States (US) [2]. BC is the second most common cause of death among the 10 causes of death due to cancer in females [2]. In the US, the mortality rate of BC is 14.8–30.8 per 100,000 people [3]. In Indonesia, BC is the most common cancer in females

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[4]. Based on histopathology results, BC is classified into in situ and invasive types. Histology studies demonstrated that 15–30% of BC is the in situ type, while 70–85% is the invasive type [1]. Among all invasive carcinomas, invasive ductal breast cancer (IDCM) is the most common subtype of invasive breast cancer (79%) [1].

Research has been conducted on genes related to cell motility, namely, familial RHO-GTPase genes including RAC1, CDC42 and RHO. Research has also been conducted on the roles of genes associated with cell migration, namely, chemokines and chemokine receptors such as CXCL12 and CXCR4 [5–10]. These studies focused on distant metastasis and general infiltration mechanisms in various cancers. The role of the familial RHO-GTPase and chemokines in the process of BC infiltration into nipple areola complex (NAC) has never been reported. Familial RHO-GTPases, particularly the RAC (RAC1) and RHO (RHOA) proteins, are involved in cell motility. The RAC1 protein facilitates the formation of lamellipodia and membrane ruffles while the RHOA protein regulates actomyosin contractility via cell body contraction [5, 7, 11]. During this process, cell migration occurs and the formation of filopodia, lamellipodia, stress fibers and actomyosin contractility is initiated. *Actomyosin* contractility requires actin reorganization to trigger cell movement. Control of actin reorganization is regulated by RAC1 and RHOA [5, 7, 9, 11]. The chemokine receptor CXCR4 and ligand CXCL12 determine the destination metastatic site of a migrating cancer cell [6, 8, 9].

This study was aimed at determining the role of these proteins as risk factors for BC infiltration into the NAC. These results may indicate an NAC skin-sparing mastectomy (NSM) operation in operable BC cases.

Materials and methods

The accessible population in this study was the OBC patient grade I, IIA, and IIB with or without infiltration into the NAC (malignant ductal epithelial cells type) where radical mastectomy had been performed in Dr. Soedarso Hospital, Pontianak and Al Ihsan Hospital, Baleendah, Bandung, from January 2006 to June 2013. Immunohistochemistry (IHC) analysis was done using anti-RAC1, RHOA or CXCR4 antibodies. Staining was performed using a streptavidin–biotin method as described previously [12]. In brief, paraffin blocks were processed in 4-µm-thick sections and were then incubated with fresh 0.3% hydrogen peroxide in methanol for 30 min at room temperature. After rinsing in phosphate-buffered saline (PBS; pH 7.4), nonspecific binding sites were blocked by incubating with 10% normal serum for 30 min. The specimens were then incubated with anti-RAC1 (ab33186, Abcam, USA), RHOA (ab54835, Abcam) or CXCR4 (ab2074, Abcam) monoclonal antibodies. The staining was scored semi-quantitatively as follows: total staining was scored as the staining intensity (on a scale of 0, 1 + to 4 +) and distribution of staining; level 4 + was the strongest and 1 + the lowest-staining intensity, and 0 for negative-staining. A distribution of < 20% was scored 1, 20–50% was scored 2, 50–80% was scored 3 and > 80% was scored 4. Statistics analysis was performed using SPSS, ver. 16.0. A Chi-square and correlation regression test was performed to determine the correlation among these proteins. p values of < 0.05 were significant.

Results

Characteristics of the research subjects were based on age, histopathology grade and stage. Chi-square test revealed that age ($p = 0.431$), histopathology grade ($p = 0.240$) and stage ($p = 0.314$) were not statistically significant; characteristics based on research subjects are homogenous. The nipple tumor distance in the NAC (+) patients was 1.71 ± 0.62 cm and in the NAC (−) patients was 2.51 ± 0.95 cm. The histology score (HS, [12]) was used for the analysis of results by the following criteria: positive intensity value added to 1 and then multiplied by the positive distribution value ($HS = [I + 1] \times d$). The cut-off point was determined using a receiver operating characteristics (ROC) curve on both groups. In the positive NAC group, staining intensity was strongly positive, indicating high expression of all three proteins (RAC1, RHOA and CXCR4; Figs. 1, 2 and 3), especially with RAC1 (85%). In positive NAC with expression of all three proteins, RAC1 had a positive distribution prevalence of more than 80% (82.5%). In positive NAC, the histoscore value prevalence score from all three proteins is 16, and RAC1 is the highest (82.5%). Therefore, RAC1 protein expression prevalence is indicated by strong positive staining intensity, positive tumor cell distribution > 80% and the highest histoscore (> 16).

The cut-off point results based on the ROC curve show that the prevalence for all three proteins in both positive and negative NAC are 93.8% for RAC1, 70% for RHOA and 78.8% for CXCR4. If only positive NAC cases are analyzed, the prevalence values obtained for RAC1, RHOA and CXCR4 are 100, 87.5 and 92.5%, respectively. If only negative NAC cases are analyzed, the prevalence values obtained for RAC1, RHOA and CXCR4 are 87.5, 62.5 and 60%, respectively. Thus, RAC1 prevalence is the highest compared to RHOA and CXCR4 in positive NAC, negative NAC, NAC positive only and NAC negative only. RHOA and CXCR4 prevalence is the highest in the NAC positive group. Furthermore, the results of the cut-off point protein expression for RAC1, RhoA, and CXCR4 associated with NAC (data not shown). For the IHC examination of RAC1, RhoA and CXCR4 protein expression, the cut-off

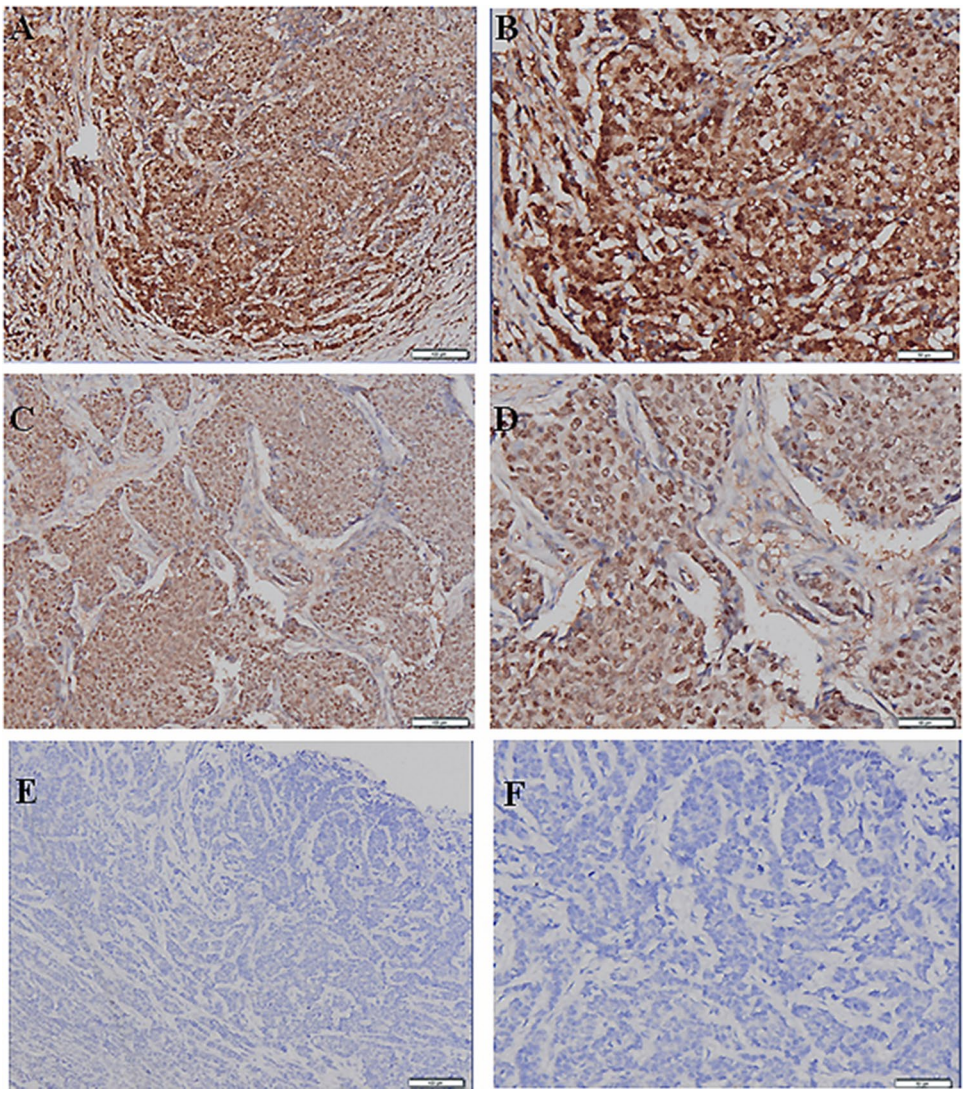

Fig. 1 RAC1 immunohistochemistry stain on tumor mass. **a** Strong RAC1 protein expression, magnification ×100. **b** Strong RAC1 protein expression, magnification ×200. **c** Positive RAC1 protein expression, magnification ×100. **d** Positive RAC1 protein expression, magnification ×200. **e** Negative RAC1 protein expression, magnification ×100. **f** Negative RAC1 protein expression, magnification ×200

points are RAC1 > 12, RhoA ≥ 1 and CXCR4 > 2. RAC1 protein expression was called (+) when the histoscore value was > 12 and (−) if the value was ≤ 12. The histoscore was called RhoA protein expression (+) when the value of the histoscore was ≥ 1 and (−) if the value was 0. The histoscore was called RAC1 protein expression (+) when the value of the histoscore was > 2 and (−) if the value of the histoscore was ≤ 2. The protein expression of RAC1, RhoA and CXCR4 and their significant relationships with the occurrence of the infiltration of BC to the NAC, with $p < 0.001$ for RAC1, $p < 0.001$ for RhoA and $p = 0.001$ for CXCR4. The magnitude of the risk for the occurrence of the infiltration of BC on the NAC with RAC1 expression (+) is 5.76x when compared to the expression of RAC1 (−). The magnitude of the risk for the occurrence of infiltration BC at the NAC with RhoA expression (+) is 7.00× when compared to the expression of RhoA (−). The magnitude of the risk of infiltration BC at the NAC with the expression of CXCR4 (+) is 6.33× compared to the expression of CXCR4 (−). The prevalence in the case group or NAC (+) group for RAC1 was 82.5% (33/40); RhoA prevalence was 87.5% (35/40) and CXCR4 prevalence was 87.5% (35/40). Table 1 shows the prevalence of RAC1 expression (+) with RhoA (+) as 75% (30/40). The interaction between RAC1 and RhoA expression was significantly associated with the occurrence of infiltration OBC to the NAC ($p < 0.001$) and had a risk of infiltration into the NAC of 17.14× compared to RAC1 and RhoA expression (−).

The interaction between RAC1 and CXCR4 expression is shown in Table 2, while the interaction between RHOA and CXCR4 expression is shown in Table 3. Table 2 shows that the interaction between RAC1 and CXCR4 expression that leads to OBC infiltration into the NAC is statistically significant ($p < 0.001$) and is 30.93 times higher compared to negative RAC1 and CXCR4 expression. Table 3 shows that the interaction between RHOA and CXCR4 expression

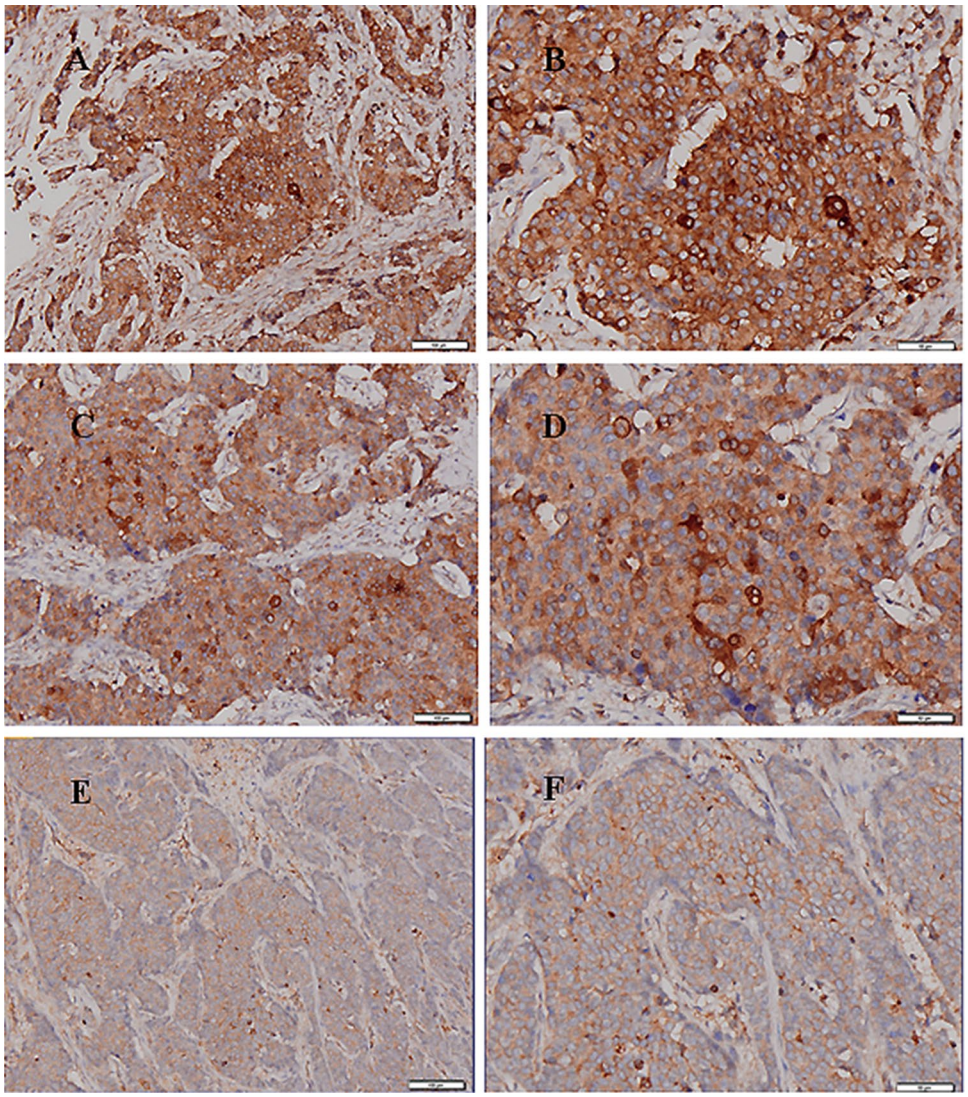

Fig. 2 RHOA immunohistochemistry stain on tumor mass. **a** Strong RHOA protein expression, magnification ×100. **b** Strong RHOA protein expression, magnification ×200. **c** Positive RHOA protein expression, magnification ×100. **d** Positive RHOA protein expression, magnification ×200. **e** Negative RHOA protein expression, magnification ×100. **f** Negative RHOA protein expression, magnification ×200

that leads to OBC infiltration into the NAC is statistically significant ($p < 0.001$) and is 10.21 times higher compared to negative RHOA and CXCR4 expression. Moreover, Chi-square test was used to determine the significance between the three variables in relation to OBC infiltration into the NAC. The interaction between RAC1, RHOA and CXCR4 is illustrated in Table 4. Positive RAC1, RHOA and CXCR4 protein expression increased OBC infiltration into the NAC by 23.69 times compared to negative expression.

Discussion

Out of 310 OBC cases in our preliminary study, there are 95 cases of infiltration into the NAC with 35% prevalence of BC infiltration into the NAC. This is similar to research conducted by Garcia-Etienne CA, 2006, who reported a 0–58% prevalence of BC infiltration into the NAC in IDCM [13]. Age, grade and stage are non-significant factors, in accordance with the findings of Laronga et al. and Simmons et al. [14, 15]. However, these results disagree with those shown by Lambert et al. [16], who claimed that grade increases the risk of BC infiltration into the NAC, and Vyas et al. [17], who claimed that stage increases the risk of BC infiltration into the NAC. RAC1 expression has the highest positive distribution value in BC cells in the NAC (82.5%) with a positive distribution value of more than 80%, while the value is only 40% in RAC1 and 35% in CXCR4. Thus, RAC1 expression is more sensitive in BC compared to RHOA and CXCR4 expression.

Migration and infiltration of cancer occur due to cytoskeletal modifications. Actin polymerization commences after activation of the ARP2/3 complex and LIM kinase (LIMK). ARP2/3 and LIMK are activated by effector familial proteins called Wiskott–Aldrich syndrome protein (WASP) and p21-activated kinase (PAK). WASP and PAK are activated

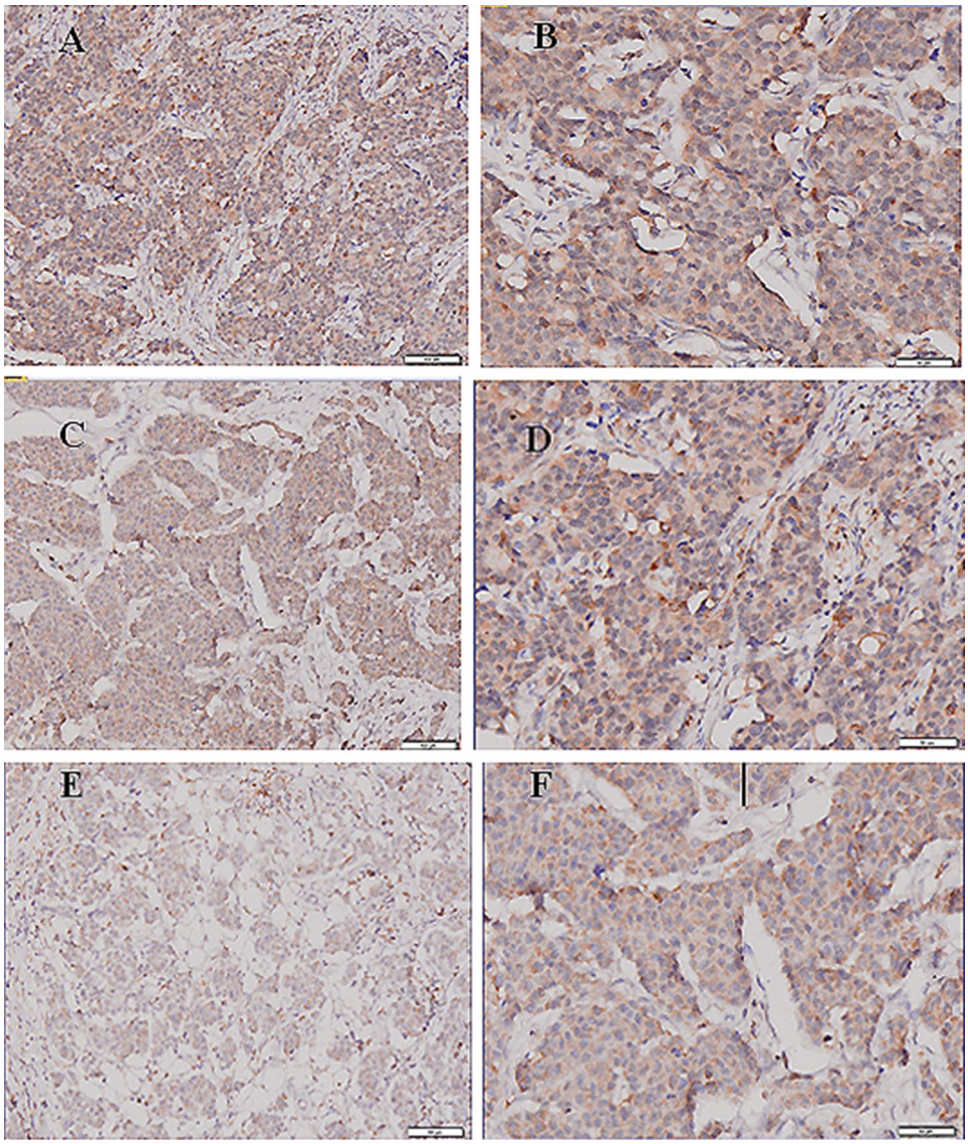

Fig. 3 CXCR4 immunohistochemistry stain on tumor mass. **a** Strong CXCR4 protein expression, magnification ×100. **b** Strong CXCR4 protein expression, magnification ×200. **c** Positive CXCR4 protein expression, magnification ×100. **d** Positive CXCR4 protein expression, magnification ×200. **e** Negative CXCR4 protein expression, magnification ×100. **f** Negative CXCR4 protein expression, magnification ×200

Table 1 RAC1 and RHOA protein expression interaction in relation with OBC infiltration into NAC

RAC1 expression	RHOA expression	Group		POR (95% CI)
		NAC (+) ($n=40$)	NAC (−) ($n=40$)	
Positive (> 12)	Positive (≥ 1)	30	14	17.14 (3.07–125.66)
	Negative	3	4	6.00 (0.52–83.95)
Negative	Positive (≥ 1)	5	6	6.67 (0.78−69.97)
	Negative	2	16	1.0

$\chi^2 = 16{,}94$; $p < 0.001$

by RAC1 and CDC42 [18]. In this study, RAC1 prevalence is 93.8% (75/80). Table 2 demonstrates that RAC1 is an influencing factor that leads to BC infiltration into the NAC with $p < 0.001$ and POR: 5.76 (95% CI 2.06–16.08). Infiltration was 5.76 times higher with positive RAC1 expression compared to negative RAC1 expression. RAC1 prevalence in this study is similar to what was described by Schnelzer et al. [5] and higher compared to what was described by Engers et al. [19]. This difference occurs due to the different cell types that were examined since RAC1 activation is related to malignancy and invasion [20, 21]. Engers et al. conducted research on prostate cancer cells, while this study

Table 2 RAC1 and CXCR4 protein expression interaction in relation with OBC infiltration into NAC

RAC1 expression	CXCR4 expression	Group		POR (95% CI)
		NAC (+) ($n=40$)	NAC (−) ($n=40$)	
Positive (> 12)	Positive (> 2)	29	15	30.93 (3.62–686.89)
	Negative	4	3	21.33 (1.30–756.95)
Negative	Positive (> 2)	6	6	16.00 (1.32–438.64)
	Negative	1	16	1.0

$\chi^2=17.83$; $p<0.001$

Table 3 CXCR4 and RHOA protein expression interaction in relation with OBC infiltration into NAC

CXCR4 expression	RHOA expression	Group		POR (95% CI)
		NAC (+) ($n=40$)	NAC (−) ($n=40$)	
Positive (> 2)	Positive (≥ 1)	33	14	10.21 (2.19–54.17)
	Negative	2	7	1.24 (0.11–13.16)
Negative	Positive (≥ 1)	2	6	1.44 (0.12–16.07)
	Negative	3	13	1.0

$\chi^2=18.71$; $p<0.001$

Table 4 RAC1, RHOA and CXCR4 protein expression interaction in relation with OBC infiltration into NAC

RAC1 expression	RHOA expression	CXCR4 expression	Group		POR (95% CI)
			Case ($n=40$)	Control (n = 40)	
(+)	(+)	(+)	28	13	23.69 (2.61–544.86)
(+)	(+)	(−)	2	1	22.00 (0.59-4331.48)
(+)	(−)	(+)	1	2	5.50 (0.0-394.08)
(+)	(−)	(−)	2	2	11.00 (0.41–601.69)
(−)	(+)	(+)	5	1	55.00 (1.98-8892.65)
(−)	(+)	(−)	0	5	–
(−)	(−)	(+)	1	5	2.20 (0.0–105.91)
(−)	(−)	(−)	1	11	1.0

χ^2; $p<0.001$

is related to BC cells. This study also showed that RAC1 increases the risk BC infiltration into the NAC by 5.76 times [19].

Migration and infiltration of BC cells requires *actomyosin* contraction. Contraction of *actomyosin* is activated by myosin light chain kinase (MLCK), RHO effector RHO kinase (ROCK) and mammalian diaphanous (mDIA). ROCK protein and mDIA are activated by RHO [22]. Fritz et al. reported that RHOA expression was extremely high and almost 50 times higher in BC cells compared to normal breast cells [23]. The prevalence of positive RHOA is 70% (56/80). Table 2 shows that RHOA is a factor that facilitates BC infiltration into the NAC with $p<0.001$ and POR: 7.00 (95% CI 2.06–16.08). RHOA prevalence in this study is lower than what was reported by Bellizi et al. [24]. This difference occurs because there were no standard criteria to interpret IHC examination; therefore, research centers developed their own criteria with varying outcomes. This study showed that RHOA protein expression is higher compared to gastric cancer cells [25], renal cell cancer [26], and colon cancer [27]. This difference may be due to different cancer cells yielding different expression levels. Infiltration was 7.00 times higher with positive RHOA expression compared to negative RHOA expression [28].

Cancer cells may express chemokine receptors and respond to chemokine gradients [26, 27]. CXCL12 is the only CXCR4 ligand [8]. CXCR4 interaction with CXCL12 plays an important role in the cell migration pathway [29]. Muller et al. 2001, claimed that the expression of the CXCR4 receptor was particularly high in BC cells and at the metastatic site [6]. BC cells with high CXCR4 expression increase the risk of lymph node metastasis [6,

8]. The prevalence of positive CXCR4 in this study was 80% (64/80). Table 2 indicates that CXCR4 influences BC infiltration into the NAC with $p = 0.001$ and POR: 6.33 (95% CI 20.6–16.08). Infiltration was 6.33 times higher with positive CXCR4 expression compared to negative CXCR4 expression. CXCR4 prevalence in this study is lower compared to Wang et al. who studied renal cell carcinoma [30], higher than in Li et al. who studied hepatocellular carcinoma [31], and higher than in Kodama et al. who studied cervical cancer [32]. This difference occurs because (I) the organs that were examined were inconsistent and provided varying prevalence, and (II) there were no standard criteria to interpret IHC examination, which allowed research centers to develop their own criteria with various outcomes. Infiltration was 6.33 times higher with positive CXCR4 expression compared to negative RHOA expression.

The interaction between RAC1 and RHOA expression in relation to NAC infiltration in BC was analyzed using a bivariate Chi-square test (Table 3). If RAC1 and RHOA expression were both positive, the POR obtained from the analysis was 17.14; 95% CI 3.07–125.66 ($p < 0.001$). This shows that positive RAC1 and RHOA expression influence OBC infiltration into the NAC. Infiltration was 17.14 times higher with positive RAC1 and RHOA expression compared to negative RAC1 and RHOA expression. The interaction between RAC1 and CXCR4 expression in relation to OBC infiltration into the NAC was analyzed using a bivariate Chi-square test. If RAC1 and CXCR4 expression were both positive, the POR obtained from the analysis was 30.93; 95% CI 3.62–686.89 (Table 4). This shows that positive RAC1 and CXCR4 expression influence OBC infiltration into the NAC. Infiltration was 30.93 times higher with positive RAC1 and CXCR4 expression compared to negative RAC1 and CXCR4 expression. Therefore, positive RAC1 and CXCR4 expression play a major role in OBC infiltration into the NAC. The interaction between RHOA and CXCR4 expression in relation to OBC infiltration into the NAC was analyzed using a bivariate Chi-square test. If RHOA and CXCR4 expression were both positive, the POR obtained from the analysis was 10.21; 95% CI 2.19–54.17 (Table not shown). This shows that positive RHOA and CXCR4 expression influenced OBC infiltration into the NAC. Infiltration was 10.21 times higher with positive RHOA and CXCR4 expression compared to negative RHOA and CXCR4 expression. Furthermore, Chi-square was also used to analyze the magnitude of OBC infiltration into the NAC if the expression of all studied proteins was positive. If the expression levels of RAC1, RHOA and CXCR4 proteins were all positive, the POR obtained from the analysis was 23.69; 95% CI 2.61–544. Infiltration was 23.69 times higher with positive RAC1, RHOA and CXCR4 expression compared to negative RHOA and CXCR4 expression.

Conclusion

Our study shows that the expression of the RAC1, RHOA, and CXCR4 proteins influences BC infiltration into the NAC. RAC1 (+) increases the effect 5.76 times, RHOA (+) increases the effect 7.00 times, and CXCR4 (+) increases the effect 6.33 times compared to the negative control. Infiltration of KPO into the NAC via the interaction of these proteins was 23.69 times higher [POR: 23.69; 95% CI 2.61–544.86 ($p < 0.001$)] than without RAC1, RHOA, and CXCR4 expression. Thus, the interactions of RAC1, RHOA, and CXCR4 protein expression lead to BC infiltration into the NAC.

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Author contributions YH, DA examined, treated and followed up the patient. AF, DI, DH wrote the manuscript. BSH evaluated pathological result. All authors read and approved the final manuscript.

Compliance with ethical standards

Ethical approval This study protocol was approved by Faculty of Medicine Ethics Committee Review Board, Universitas Padjadjaran and all study participants gave informed consent. All authors hereby declare that all patients have been examined in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

Informed consent All authors declare that written informed consent was obtained from the patient (or other approved parties) for publication of this study and accompanying images.

Availability of data and materials section Authors declare that the data will not be shared, since it is confidential.

Conflict of interest Authors have declared that no conflict of interest exists.

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