Profiling the expression of pro-metastatic genes in association with the clinicopathological features of primary breast cancer

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Abstract

Background: Metastasis accounts for ninety percent of breast cancer (BrCa) mortality. Cortactin, Ras homologous gene family member A (RhoA), and Rho-associated kinase (ROCK) raise cellular motility in favor of metastasis. Claudins (CLDN) belong to tight junction integrity and are dysregulated in BrCa. Thus far, epidemiologic evidence regarding the association of different pro-metastatic genes with pathological phenotypes of BrCa is largely inconsistent. This study aimed to determine the possible transcriptional models of pro-metastatic genes incorporate in holding the integrity of epithelial cell–cell junctions (CTTN, RhoA, ROCK, CLDN-1, CLDN-2, and CLDN-4), for the first time, in association with clinicopathological features of primary BrCa.

Methods: In a consecutive case-series design, 206 newly diagnosed non-metastatic eligible BrCa patients with histopathological confirmation (30–65 years) were recruited in Tabriz, Iran (2015–2017). Real-time RT-PCR was used. Then fold changes in the expression of target genes were measured.

Results: ROCK amplification was associated with the involvement of axillary lymph node metastasis (ALNM; ORadj. = 3.05, 95%CI 1.01–9.18). Consistently, inter-correlations of CTTN-ROCK (β = 0.226, P < 0.05) and RhoA-ROCK (β = 0.311, P < 0.01) were determined among patients diagnosed with ALNM+. The overexpression of CLDN-4 was frequently observed in tumors identified by ALNM+ or grade III (P < 0.05). The overexpression of CTTN, CLDN-1, and CLDN-4 genes was correlated positively with the extent of tumor size. CTTN overexpression was associated with the increased chance of luminal-A positivity vs. non-luminal-A (ORadj. = 1.96, 95%CI 1.02–3.77). ROCK was also expressed in luminal-B BrCa tumors (P < 0.05). The estrogen receptor-dependent transcriptions were extended to the inter-correlations of RhoA-ROCK (β = 0.280, P < 0.01), ROCK-CLDN-2 (β = 0.267, P < 0.05), and CLDN-1-CLDN-4 (β = 0.451, P < 0.001).

Conclusions: For the first time, our findings suggested that the inter-correlations of CTTN-ROCK and RhoA-ROCK were significant transcriptional profiles determined in association with ALNM involvement; therefore the overexpression of ROCK may serve as a potential molecular marker for lymphatic metastasis. The provided binary transcriptional profiles need more approvals in different clinical features of BrCa metastasis.

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Background
Breast cancer (BrCa) is globally the most common malignancy in women. BrCa is a heterogeneous disease with incident rate of 46.3 per 100,000 and mortality rate of 13.0 per 100,000 in 2018 worldwide, according to GLOBOCAN [1]. BrCa incidence in developing countries is increased due to cultural transition toward a sedentary lifestyle, Western diet, and increased rate of smoking, urbanization, and air pollutions [2–4]. It is accepted that estrogen leads to BrCa progression. Estrogen signaling is a therapeutic target for BrCa [5]; identifying pro-metastatic gene expression in different molecular subtypes (hormone receptors) with unique prognostic features may help elucidating new more personalized therapies. Furthermore, some important studies have documented evidence about preclinical diagnostic markers from predisposing mutations to raise the risk of BrCa [6], and the effect of some interventions on molecular targets have been assessed in solid tumors [7–10]. These could support lacking a consensus to support pro-metastatic genes in association with advanced features. Metastasis is considered for 90% of BrCa mortality which involves a complex multi-stage process, initially breaking away of tumor cells from primary tumor (dissociation step), degrading the proteins incorporate in the integrity of extracellular matrix (invasion step), transmigrate through vascular and/or lymph vessel (intravasation and extravasation steps), and cell-to-cell and cell–matrix adhesions with certain affinity to make organ-specific target metastasis (organotropism) [11].

Cortactin regulates actin cytoskeleton arrangement—a prerequisite for metastasis progressions—by binding to actin-related protein complex and facilitating releasing activated Wiskott Aldrich syndrome proteins [12]. Cortactin is an important regulator of cancer cell motility and mesenchymal movement [13]. Invadopodia, forming cellular actin-based protrusions, is mediated by cortactin activation and accompanies the invasion of cancer cells to the mesenchymal layer [14]. The overexpression of CTTN was associated with lymph node metastasis [15–19], advanced histologic grades [16, 20, 21], and larger tumor size [15, 16] in various cancers suggesting that cortactin might have prognostic impacts on different cancers but less paid attention to BrCa. One study reported a significant correlation between the protein expression of cortactin and lymphatic metastasis of breast tumors [22]. Therefore, the CTTN expression in association with histologic grade and tumor size of BrCa is largely missing. Of the few earlier reports on cortactin expression in BrCa regarding molecular subtypes [23, 24], a meta-cohort of primary BrCa reported CTTN overexpression in hormone receptor-positive samples [24].

There is a bifunctional activity between cortactin and Ras homologous gene family member A/Rho-associated kinase (RhoA/ROCK) complex in integration for enhancing actin stress fiber formation [12, 25]. RhoA belongs to the small GTPase family [25]. It triggers cell motility and amoeboid movement via the extensive formation of actin stress fiber and actomyosin contractility regulation [25]. ROCK activity—a major downstream effector of RhoA—is to stabilize actin filament and phosphorylate myosin light chain to eventually raise reforming rates of actomyosin contractility [26]. RhoA or ROCK overexpression predicts shorter survival rates of BrCa [27, 28]. RhoA overexpression was observed in advanced histologic grade [29], larger tumor size [27], and stages II–III of BrCa [30]. ROCK overexpression was correlated with nodal involvement and advanced histologic grade of BrCa [28, 31]. Significant results were not reported regarding the association of RhoA and ROCK expression with lymphatic metastasis and tumor size of BrCa, respectively. Studies could rarely provide evidence to show the association of molecular subtype of BrCa and the expression of RhoA or ROCK.

Claudins (CLDNs)—membrane transport proteins—have critical functions in forming and maintaining cell-to-cell adhesion complexes, so-called tight junctions [32]. CLDNs generally connect to actin cytoskeleton, and their functions may be regulated by Rho/ROCK pathway [33]. CLDNs dysregulation is documented in epithelial-derived cancers [32]. Different isoforms of CLDNs present in a tissue-specific manner. Their various functions highly depend on cell’s microenvironment [34]. The protein expression of CLDN-1 was reported to be associated with poor prognosis [35], larger tumor size [36], and advanced histologic grade of BrCa [36, 37]. However, positive or negative protein expression of CLDN-1 was reported to be correlated to the lymph node metastasis of BrCa [36, 38]. While the positive protein expression of CLDN-2 was associated with poor prognosis [38, 39], its loss of expression was related to nodal involvement of BrCa [34]. No study reported a significant correlation between CLDN-2 expression and histologic grade and tumor size of BrCa patients. Several reports indicated a positive protein expression of CLDN-4 connected to poor prognosis [37, 40], advanced grade [37, 40–43], and lymphatic metastasis [19, 41] of BrCa. However, positive or negative protein expression of CLDN-4 was reported to be correlated to a larger tumor size of BrCa [37, 43]. CLDNs expression was dependent on the molecular...
subtypes of breast tumors [35–38, 40, 41, 43, 44]. The data on the association of CLDNs expression and clinicopathological parameters remains inconclusive.

Sufficient information is not available to interpret pro-metastatic genes interaction in association with pathologic features in non-metastatic conditions. Therefore, this study aimed (i) to assess the correlation of pro-metastatic genes – CTTN, RhoA, ROCK, CLDN-1, CLDN-2, and CLDN-4 – with clinicopathological parameters, (ii) to determine the inter-correlation of pro-metastatic genes in primary BrCa.

Methods
Study population
This study is a part of ongoing large prospective cohort of consecutive case-series of BrCa patients-Breast Cancer Risk and Lifestyle (BCRL)- who were histopathologically diagnosed with primary malignancy. The BCRL is a multicenter study designed to assess lifestyle-related factors in association with BrCa risk prevention, regional to Northwestern Iran. The present study is a part of this cohort with ongoing recruitment began in May 2009. Participants with newly diagnosed and histologically confirmed BrCa (N=206), who were admitted to the surgical wards of Nour-Nejat Hospital, Shams Hospital, Shahid Ghazi Educational-Oncology Hospital, and several oncology clinics located in Tabriz, Iran from May 2015 to January 2017. These are referral hospitals for oncologic surgeries with patients from different Northwestern Iran provinces (East and West Azerbaijan, Ardabil, Hamadan, and Zanjan). Participants were recruited from BrCa candidates before mastectomy surgery. The participants were 30-65 years old at the time of BrCa diagnosis. Eligible participants were mostly recruited from pre-menopausal women who had lymph node(s) positivity, invasive ductal carcinoma (IDC), and stages I-III. Other inclusion criteria mainly consisted of a completed written informed consent form and no subjective medical history, including benign breast diseases, other malignancy, and any oncologic surgery. Exclusion criteria were reported in our previous reports [3, 4, 45, 46] which were depicted in Fig. 1. Family history of breast and other cancers in first- and second-degree relatives were asked to lay out related pedigree analysis. Anthropometric measurements were examined as well.

Pathologic data
Histopathologic data, including tumor size, histological subtype (IDC and non-ductal carcinoma), axillary lymph node metastasis (ALNM), and histological tumor grade, were obtained from objective medical records. The tumor size was considered the greatest diameter of tumor [47] Evaluating the histologic grades was determined by reviewing the stained microtones of paraffin-embedded tumor samples according to the Nottingham combined grading system to detect grades based on tubule formation, nuclear grade, and mitotic activity [47]. Immunohistochemical staining was carried out for human epidermal growth factor receptor-2 (HER2), estrogen receptor (ER), and progesterone receptor (PR) (Fig. 2). For HER2 positivity, the membrane and cytoplasmic staining ≥ 10% of breast tumor cells were considered weak or high intensity [47]. Immunohistochemistry results obtained by nuclear antibody staining when accounted for above 1% of tumor cells indicated the positivity of ER and PR staining [48]. Molecular subtypes were classified based on the protocol summarized by Wu et al. [47]. Clinical staging was defined according to the American Joint Committee on Cancer; 8th BC staging guidelines [49]. Neither BrCa patients with contralateral ALNM classified as distant metastases (M1) nor BrCa patients with distant metastasis (M1; stage IV) were recruited [49]. Some pathological data were not available due to missing.

Quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR)
Before mastectomy surgery, fresh frozen tumor tissue and normal adjacent tissue were collected. Surgical tumor tissue section (core biopsy) consisted of 85% tumor cells with microscopic examinations [50]. QIAzol (Qiagen, USA) was applied to extract total mRNA and then evaluated using NanoDrop 2000 (Thermo Scientific, Germany), accounting for the absorbance at 260/280 nm. Total mRNA was converted into cDNA using Light Cycler 480II (Roche, Germany). Materials used for PCR were composed of 12.5 μl from 10 × SYBER Green Master Mix (Nanohelix, South Korea), each primer solution (~ 10 pmol/μl), template cDNA (~100–200 ng/μl), and DEPC-treated water. PCR steps included an initial denaturation (15 min at 95 °C), followed by 40 cycles of denaturation (24 s at 95 °C) and annealing (35 s at 62 °C). Each sample was amplified in duplicate reactions. The amplification of hypoxanthine–guanine phosphoribosyltransferase was as an internal control (Additional file 1: Figure S1). Fold changes in the expression of target genes (CTTN, RhoA, ROCK, CLDN-1, CLDN-2, and CLDN-4) were calculated using a 2ΔΔct formula [51]. PCR primers for relevant genes were listed in Additional file 2: Table S1.

Statistical analysis
A sample size including 158 subjects was calculated based on information provided by Dales et al. [52] regarding type 1 error (alpha) at 0.05 and the power of
Invitation at surgery ward (n= 614)

Participants responded to invitation (n= 532)

Excluded:
- History of chemotherapy (n= 32)
- History of other malignancies (n= 20)
- History of oncologic surgeries (n= 37)
- History of benign tumors (n= 43)

Excluded:
- Pregnant (n= 14)
- Breastfeeding (n= 15)
- Morbid obesity (n= 7)
- Excluding medicine (n= 19)
- Stage IV (n= 114)

Eligible participants (n= 231)

Excluded:
- Histopathological disconfirmation of BC (n= 25)

Gene expression analyzed (n=206)

Fig. 1 Flow chart diagram for the selection of study participants

Fig. 2 Immunohistochemical protein expression of human epidermal growth factor receptor 2 (HER2), estrogen receptor (ER), and progesterone receptor (PR). The original magnification was X400
analysis ($1 - \beta$) at 90%. Outlier data were detected using the box plots. Kolmogorov–Smirnov test and histogram plots were carried out to assess the normal distribution of continuous variables. After using Chi square test, the correlation between two sets of categorical variables was interpreted. Fold change in the expression of gene was compared among sub-categories of clinicopathological (molecular subtypes, histologic grades, tumor size, and ALNM) by conducting the one-way analysis of covariance (ANCOVA) set at posthoc Bonferroni method, and the results were represented by bar diagram. Linear regression analysis was performed to present standardized $\beta$-coefficients ($\beta$) among genes in certain clinical stages (I and II–III) and binary status of hormone receptors, and ALNM features. Also, scatter plots were illustrated to show the correlations between targeted genes and tumor size. Standardized $\beta$ out of linear regression analysis was accompanied by scatter plots in crude ($\beta$) and adjusted models ($\beta_{adj}$). Fold changes in the expression of genes were dichotomized using (1) median values, and (2) cutoff points determined by plotting receiver operating characteristic (ROC) curve based on ALNM status (as reference). Odds ratio (OR) and 95% confidence interval (95% CI) were obtained by logistic regression analysis to explore interesting genes as independent determinants of clinicopathological outcomes. The primary criteria for selecting a confounder in a model were identifying a significant univariate analysis concerning dependent variable. A certain model was identified for each gene using multivariate logistic regression analysis. The adjusted confounding factors were listed in Additional file 3: Table S2. Statistical analyses were performed using SPSS software, version 16 (SPSS Inc., USA). All two-tailed $P$-values < 0.05 were considered significant.

**Results**

The clinicopathological characteristics of 206 patients were summarized in Table 1. The mean age in diagnosing these patients was 46.65 ± 8.61 years. Patients were frequently younger than 24 years at first pregnancy (60.6%, $P < 0.01$) and were older than 13 years at menarche (80.1%, $P < 0.001$). They were pre-menopause (66.5%) and histopathologically identified with IDC (92.0%), ER+ (87.3%), PR+ (85.1%), and HER2- (79.6%) in the total study population ($P < 0.001$). Histologically, the most frequent dimension of the tumor was T2 (2 cm < size ≤ 5 cm) (61.3%, $P < 0.001$). Stage II BrCa was more frequent among the participants (65.7%). Histologic grade II tumors (67.4%) and ALNM involvement (65.2%) were the most observed histological features ($P < 0.001$). A significant agreement existed between BrCa frequency diagnosed with ALNM and lymphatic invasion status ($P < 0.001$), indicating the acceptable accuracy of data represented as ALNM status (Additional file 4: Table S3).

Figure 3 illustrates bar diagrams comparing the fold changes in the expression of relevant genes among categorical dependent factors including tumor size ($\leq 2$, and $> 2$ cm), ALNM (+/-), histologic grades (I, II, and III), and molecular subtypes. Larger tumor size ($> 2$ cm) showed higher expression levels of $CTTN$, $CLDN-1$, and $CLDN-4$ than those in smaller tumors ($P < 0.05$). Over-expressions of $ROCK$ and $CLDN-4$ were observed in ALNM+ tumors more than BrCa counterparts lacking ALNM ($P < 0.05$). The expression levels of $CTTN$ among the patients with grade II were higher than grade I ($P < 0.05$). There was an increasing trend in $CLDN-4$ expression level among rising grades ($P < 0.05$). Of luminal A tumors, the fold change in the expression level of $ROCK$ was found out less than those in luminal B and triple-negative tumors ($P < 0.01$).

The scatter plots indicating the correlations among the genes of interest and tumor size were shown in Fig. 4. $CTTN$ overexpression ($\beta_{adj.} = 0.253$, $P < 0.05$), $CLDN-1$ ($\beta_{adj.} = 0.345$, $P < 0.01$), and $CLDN-4$ ($\beta_{adj.} = 0.338$, $P < 0.01$) were significantly correlated to the larger tumor dimension in the models adjusted for potential covariates.

OR and 95% CI estimated to represent the associations between the expression levels of genes and ALNM status (Table 2) and tumor grades (Table 3) using unadjusted (crude) and multivariate-adjusted models. According to high expression levels of $ROCK$ in lymph node-positive (Fig. 3), $ROCK$ overexpression was significantly associated with ALNM+ after adjustments for potential covariates (OR $\text{Median-based cutoff} = 3.05$, 95%CI 1.01–9.18) (Table 2). The overexpressions of $CTTN$ (OR $\text{ROC-based cut-off} = 4.33$, 95%CI 1.64–11.43) and $ROCK$ (OR $\text{ROC-based cut-off} = 2.92$, 95%CI 1.18–7.24) were associated with developed grade II breast carcinoma (Table 3). Multivariate adjusted models showed positive associations between $CTTN$ (OR $\text{ROC-based cutoff} = 5.08$, 95%CI 1.75–14.69) and $ROCK$ (OR $\text{ROC-based cutoff} = 2.66$, 95%CI 1.14–7.14) for grade II tumors vs. considering grade I as reference (Table 3). Moreover, the overexpressions of $CTTN$ and $ROCK$ were associated with grade III in crude ($CTTN$: OR $\text{ROC-based cutoff} = 3.90$, 95%CI 1.10–13.81; $ROCK$: OR $\text{ROC-based cutoff} = 4.40$, 95%CI 1.33–14.48) and adjusted (CTTN: OR $\text{ROC-based cutoff} = 5.08$, 95%CI 1.32–19.44; ROCK: OR $\text{ROC-based cutoff} = 4.22$, 95%CI 1.26–14.07) models (Table 3).

Since luminal A was the predominant sub-class of molecular subtypes in the present study, $CTTN$ overexpression was significantly associated with luminal A vs. other molecular subtypes after adjustment for related confounders (OR $\text{Median-based cutoff} = 1.96$, 95%CI 1.02–3.77) (Table 4). Tumors characterized by luminal B (vs. non-luminal B) was remarkable in tumors...
Table 1  Clinicopathological characteristics of patients with invasive BrCa (N = 206)

| Variable                     | Total patients (n) | The relative frequency (%) | P-value* |
|------------------------------|--------------------|----------------------------|----------|
| Age at diagnosis (year)      |                    |                            |          |
| Mean ± S.D.                  | 46.65 ± 8.61       |                            |          |
| < 48                         | 117                | 56.8                       | 0.051    |
| ≥ 48                         | 89                 | 43.2                       |          |
| Age at first pregnancy (year)|                    |                            |          |
| Mean ± S.D.                  | 22.11 ± 4.86       |                            |          |
| < 24                         | 120                | 60.6                       | 0.003    |
| ≥ 24                         | 78                 | 39.4                       |          |
| Age at menopause (year)      |                    |                            |          |
| Mean ± S.D.                  | 47.86 ± 4.73       |                            |          |
| Post-menopause               | 69                 | 33.5                       | <0.001   |
| Pre-menopause                | 137                | 66.5                       |          |
| Age at menarche (year)       |                    |                            |          |
| Mean ± S.D.                  | 13.52 ± 1.36       |                            |          |
| < 13                         | 40                 | 19.9                       | <0.001   |
| ≥ 13                         | 161                | 80.1                       |          |
| Tumor type                   |                    |                            |          |
| IDC                          | 172                | 92.0                       | <0.001   |
| Others                       | 15                 | 8.0                        |          |
| Tumor size (cm)              |                    |                            |          |
| Mean ± S.D.                  | 2.96 ± 1.35        |                            |          |
| T1 (size ≤ 2)                | 59                 | 31.7                       | <0.001   |
| T2 (2 < size ≤ 5)            | 114                | 61.3                       |          |
| T3 (size > 5)                | 13                 | 7.0                        |          |
| Histologic grade             |                    |                            |          |
| I                            | 39                 | 20.9                       | <0.001   |
| II                           | 126                | 67.4                       |          |
| III                          | 22                 | 11.7                       |          |
| Axillary lymph node metastasis|                   |                            |          |
| Negative                     | 64                 | 34.8                       | <0.001   |
| Positive                     | 120                | 65.2                       |          |
| Tumor stage                  |                    |                            |          |
| I                            | 55                 | 30.4                       | <0.001   |
| II                           | 119                | 65.7                       |          |
| III                          | 7                  | 3.9                        |          |
| ER                           |                    |                            |          |
| Negative                     | 23                 | 12.7                       | <0.001   |
| Positive                     | 158                | 87.3                       |          |
| PR                           |                    |                            |          |
| Negative                     | 27                 | 14.9                       | <0.001   |
| Positive                     | 154                | 85.1                       |          |
| HER2                         |                    |                            |          |
| Negative                     | 144                | 79.6                       | <0.001   |
| Positive                     | 37                 | 20.4                       |          |
| Molecular subtype            |                    |                            |          |
| Luminal A (ER±, PR±, and HER2-) | 135           | 74.6                       | <0.001   |
| Luminal B (ER±, PR±, and HER2+) | 23              | 12.7                       |          |
| HER2 rich (ER-, PR-, and HER2+) | 14              | 7.7                        |          |
| Triple-negative (ER-, PR-, and HER2-) | 9            | 5.0                        |          |

The statistically significant finding was shown in italics (P < 0.05)

N number, S.D. standard deviation, ER estrogen receptor, PR progesterone receptor, HER2 human epidermal growth factor receptor 2, IDC invasive ductal carcinoma

*The P-value was obtained by the Chi square test.

Some missing existed in demographic and clinicopathologic data
overexpressed ROCK (OR ROC-based cutoff = 2.76, 95% CI 1.07–7.11). ROCK expression levels were also associated with triple-negative status compared to non-triple negative subtypes (OR ROC-based cutoff = 6.29, 95% CI 1.27–31.11) (Table 4).

Linear regression analysis was performed to obtain correlation coefficients (β) among the genes of interest and subgroup analyses due to hormonal receptor status, ALNM feature, and clinical staging, and presented in Table 5. For the total study population, findings indicated positive associations among the expression levels of ROCK and RhoA (β = 0.246, P < 0.001), CTTN (β = 0.170, P < 0.05), and CLDN-2 (β = 0.237, P < 0.05) as dependent variables (Table 5). CLDN-1 expression levels were strongly correlated to CLDN-4 as well (β = 0.411, P < 0.001) (Table 5).

In the case of ER+ tumors (PR +/-), ROCK up-regulation was significantly correlated to RhoA (β = 0.280, P < 0.01) and CLDN-2 (β = 0.267, P < 0.05). Of tumors characterized by ER positivity, CLDN-1 was significantly associated with CLDN-4 (β = 0.411, P < 0.001) (Table 5). In ER-negative patients, the expression levels of CTTN and CLDN-2 were strongly intercorrelated (β = 0.655, P < 0.05) (Table 5).

ROCK overexpression was significantly associated with up-regulation at RhoA (β = 0.311, P < 0.01) and CTTN (β = 0.226, P < 0.05) when there was the involvement of ALNM. The expression levels of CLDN-1 and CLDN-4 were inter-correlated in sub-population defined by the presence of ALNM (β = 0.377, P < 0.001) and absent ALNM at diagnosis (β = 0.522, P < 0.001) (Table 5). In the absence of ALNM development, ROCK expression levels was strongly associated with CLDN-2 (β = 0.733, P < 0.001) (Table 5).

Of patients at stage I, ROCK up-regulation was strongly correlated to CTTN (β = 0.519, P < 0.01) and CLDN-2
Fig. 4 Scatter plots showing linear regression coefficients (standardized $\hat{\beta}$) were depicted to present correlations between fold changes in the expression of studied genes and tumor size of BrCa ($n = 186$). $\text{CTTN}$ cortactin, $\text{RhoA}$ ras homolog gene family member A, $\text{ROCK}$ rho-associated kinase, $\text{CLDN}$ claudin, $\beta_{\text{adj}}$ adjusted $\hat{\beta}$. $^a$Adjusted for abortion (number), pregnancy (number), and hip circumference (cm); $^b$Adjusted for age at diagnosis (year) and BMI (kg/m²); $^c$Adjusted for age at diagnosis (year) and waist to hip ratio; $^d$Adjusted for abortion (number), lactation (number), the age at menarche (year), and duration of oral contraceptive usage (month); $^e$Adjusted for waist circumference (cm) and age at first pregnancy (year); $^f$Adjusted for age at menarche (year), BMI (kg/m²), abortion (number) and duration of oral contraceptive usage (month). A significant result was indicated by $^*P < 0.05$. 

| Gene | $\hat{\beta}$ | $P$ | $\beta_{\text{adj}}$ | $P_{\text{adj}}$ |
|------|---------------|-----|---------------------|------------------|
| CTTN | 0.110         | 0.178 | 0.253               | 0.013            |
| RhoA | 0.054         | 0.476 | 0.068               | 0.391            |
| ROCK | -0.051        | 0.503 | 0.033               | 0.681            |
| CLDN-2 | 0.112     | 0.140 | 0.345               | 0.003            |
| CLDN-4 | 0.141     | 0.062 | 0.338               | 0.004            |
Table 2 Odds ratios (ORs) and 95% confidence intervals (95%CI) presenting associations between fold changes in the expression of CTTN, RhoA, ROCK, CLDN-1, CLDN-2, and CLDN-4 genes and axillary lymph node metastasis (ALNM) as a dependent pathological variable of primary non-metastatic BrCa-IDC (no distant metastasis) (n = 173)

| Expression levels of gene | ALNM status | Crude OR (95%CI) | Adjusted OR (95%CI) |
|--------------------------|-------------|-----------------|---------------------|
|                          | N           | Negative | Positive | P-value | Negative | Positive | Negative | Positive |
| CTTN                    |             |          |          |         |          |          |          |          |
| <2.31*                  | 72          | 22 (30.6) | 50 (69.4) | 0.282   | 1.00     | 0.77     | (0.38–1.52) | 1.00     | 0.94     | (0.46–1.92) |
| ≥2.31                   | 77          | 28 (36.4) | 49 (63.6) |          |          |          |          |          |
| <4.88**                 | 85          | 29 (34.1) | 56 (65.9) | 0.504   | 1.00     | 1.06     | (0.53–2.11) | 1.00     | 1.28     | (0.62–2.64) |
| ≥4.88                   | 64          | 21 (32.8) | 43 (67.2) |          |          |          |          |          |
| RhoA                    |             |          |          |         |          |          |          |          |
| <0.69*                  | 86          | 26 (30.2) | 60 (69.8) | 0.182   | 1.00     | 0.70     | (0.37–1.33) | 1.00     | 0.74     | (0.39–1.40) |
| ≥0.69                   | 87          | 33 (37.9) | 54 (62.1) |          |          |          |          |          |
| <0.73**                 | 87          | 26 (29.9) | 61 (70.1) | 0.155   | 1.00     | 0.68     | (0.36–1.28) | 1.00     | 0.71     | (0.37–1.35) |
| ≥0.73                   | 86          | 33 (38.4) | 53 (61.6) |          |          |          |          |          |
| ROCK                    |             |          |          |         |          |          |          |          |
| <0.83*                  | 85          | 31 (36.5) | 54 (63.5) | 0.353   | 1.00     | 1.18     | (0.63–2.23) | 1.00     | 3.05     | (1.01–9.18) |
| ≥0.83                   | 86          | 28 (32.6) | 58 (67.4) |          |          |          |          |          |
| <1.09**                 | 104         | 39 (37.5) | 65 (62.5) | 0.195   | 1.00     | 1.41     | (0.73–2.71) | 1.00     | 2.18     | (0.75–6.33) |
| ≥1.09                   | 67          | 20 (29.9) | 47 (70.1) |          |          |          |          |          |
| CLDN-1                  |             |          |          |         |          |          |          |          |
| <1.06*                  | 88          | 35 (39.8) | 53 (60.2) | 0.135   | 1.00     | 1.49     | (0.79–2.81) | 1.00     | 1.55     | (0.82–2.95) |
| ≥1.06                   | 85          | 26 (30.6) | 59 (69.4) |          |          |          |          |          |
| <0.75**                 | 37          | 15 (40.5) | 22 (59.5) | 0.284   | 1.00     | 1.33     | (0.63–2.81) | 1.00     | 1.35     | (0.63–2.87) |
| ≥0.75                   | 136         | 46 (33.8) | 90 (66.2) |          |          |          |          |          |
| CLDN-2                  |             |          |          |         |          |          |          |          |
| <1.15*                  | 45          | 12 (26.7) | 33 (73.3) | 0.273   | 1.00     | 0.57     | (0.23–1.37) | 1.00     | 0.54     | (0.21–1.37) |
| ≥1.15                   | 49          | 19 (38.8) | 30 (61.2) |          |          |          |          |          |
| <1.12**                 | 15          | 6 (40.0)  | 9 (60.0)  | 0.363   | 1.00     | 1.44     | (0.46–4.48) | 1.00     | 1.60     | (0.48–5.35) |
| ≥1.12                   | 79          | 25 (31.6) | 54 (68.4) |          |          |          |          |          |
| CLDN-4                  |             |          |          |         |          |          |          |          |
| <0.85*                  | 85          | 29 (34.1) | 56 (65.9) | 0.503   | 1.00     | 0.95     | (0.50–1.78) | 1.00     | 0.99     | (0.52–1.87) |
| ≥0.85                   | 88          | 31 (35.2) | 57 (64.8) |          |          |          |          |          |
| <0.67**                 | 67          | 28 (38.8) | 41 (61.2) | 0.229   | 1.00     | 1.34     | (0.70–2.54) | 1.00     | 1.37     | (0.72–2.62) |
| ≥0.67                   | 106         | 34 (32.1) | 72 (67.9) |          |          |          |          |          |

The statistically significant finding is shown in italic fonts (P < 0.05)

N number, OR odds ratio, CI confidence intervals, ALNM axillary lymph node metastasis, CTTN cortactin, RhoA ras homolog gene family member A, ROCK rho-associated kinase, CLDN claudin

* Data were expressed as number (%)

* Logistic regression analysis was performed

Adjusted for: Education status (illiterate/middle school/diploma/bachelor and higher) and waist circumference (< 90 cm/90 ≤ cm); Age at menarche (< 13 year/13 ≤ year) and waist circumference (< 90 cm/90 ≤ cm); Age at first pregnancy (< 24 year/24 ≤ year) and body mass index (< 24.99 kg/m²/25–29.99 kg/m²); Education status (illiterate/middle school/diploma/bachelor and higher) and body mass index (< 24.99 kg/m²/25–29.99 kg/m²); Education status (illiterate/middle school/diploma/bachelor and higher) and number of pregnancy (0–1/2–3/≥ 4); Age at menarche (< 13 year/13 ≤ year) and number of pregnancy (0–1/2–3/≥ 4)

* Chi square test was performed

* Fold changes in the expression of studied genes were categorized based on median values

** Fold changes in the expression of studied genes were categorized based on the cutoff identified by Youden's index

| Data were expressed as number (%) | Logistic regression analysis was performed | Adjusted for: Education status (illiterate/middle school/diploma/bachelor and higher) and waist circumference (< 90 cm/90 ≤ cm); Age at menarche (< 13 year/13 ≤ year) and waist circumference (< 90 cm/90 ≤ cm); Age at first pregnancy (< 24 year/24 ≤ year) and body mass index (< 24.99 kg/m²/25–29.99 kg/m²); Education status (illiterate/middle school/diploma/bachelor and higher) and body mass index (< 24.99 kg/m²/25–29.99 kg/m²); Education status (illiterate/middle school/diploma/bachelor and higher) and number of pregnancy (0–1/2–3/≥ 4); Age at menarche (< 13 year/13 ≤ year) and number of pregnancy (0–1/2–3/≥ 4) | Chi square test was performed | Fold changes in the expression of studied genes were categorized based on median values | Fold changes in the expression of studied genes were categorized based on the cutoff identified by Youden's index |
Table 3  Odds ratios (ORs) and 95% confidence intervals (95%CI) demonstrating associations between fold changes in the expression of *CTTN*, *RhoA*, *ROCK*, *CLDN-1*, *CLDN-2*, and *CLDN-4* genes and histologic grades as a dependent histopathological variable of primary non-metastatic BrCa (no distant metastasis) (n = 176)

| Expression levels of gene | Grade | N   | I   | II  | III | P-value* | I    | II   | III  | P for trend | I    | II   | III  | P for trend |
|--------------------------|-------|-----|-----|-----|-----|----------|------|------|------|-------------|------|------|------|-------------|
| CTTN                     |       |     |     |     |     |          |      |      |      |             |      |      |      |             |
| <2.31*                   | 72    | 22  | 42  | 8   | 41.1 | 0.027    | 1.00 | 3.03 | (1.30–7.08) | 3.02 | (0.93–9.82) | 0.03  | 100   | 3.37 | (1.35–8.38) | 3.69 | (1.08–12.61) |
| ≥2.31                     | 77    | 10  | 58  | 11  | 13.9 |          |      |      |      |             |      |      |      |             |
| <4.88**                  | 86    | 26  | 50  | 10  | 11.6 | 0.007    | 1.00 | 4.33 | (1.64–11.43)| 3.90 | (1.10–31.81)| 0.016 | 100   | 5.08 | (1.75–14.69) | 5.08 | (1.32–19.44) |
| ≥4.88                      | 65    | 6   | 50  | 9   | 13.8 |          |      |      |      |             |      |      |      |             |
| RhoA                     |       |     |     |     |     |          |      |      |      |             |      |      |      |             |
| <0.69*                   | 86    | 22  | 55  | 9   | 10.5 | 0.238    | 1.00 | 1.77 | (0.82–3.79) | 227  | (0.76–6.69) | 0.107 | 100   | 1.81 | (0.83–3.96) | 2.58 | (0.86–7.76) |
| ≥0.69                     | 89    | 14  | 62  | 13  | 14.6 |          |      |      |      |             |      |      |      |             |
| <0.73**                  | 87    | 22  | 55  | 10  | 11.5 | 0.305    | 1.00 | 1.77 | (0.82–3.79) | 188  | (0.64–5.51) | 0.184 | 100   | 1.81 | (0.83–3.96) | 2.14 | (0.72–6.38) |
| ≥0.73                    | 88    | 14  | 62  | 12  | 13.6 |          |      |      |      |             |      |      |      |             |
| ROCK                     |       |     |     |     |     |          |      |      |      |             |      |      |      |             |
| <0.83*                   | 85    | 21  | 55  | 9   | 10.6 | 0.347    | 1.00 | 1.66 | (0.77–3.58) | 200  | (0.66–9.99) | 0.172 | 100   | 1.65 | (0.76–3.58) | 1.90 | (0.63–5.74) |
| ≥0.83                    | 87    | 14  | 61  | 12  | 13.8 |          |      |      |      |             |      |      |      |             |
| <1.09**                  | 105   | 28  | 67  | 10  | 9.3  | 0.025    | 1.00 | 2.92 | (1.18–7.24) | 440  | (1.33–14.48)| 0.011 | 100   | 2.86 | (1.14–7.14) | 4.22 | (1.26–14.07) |
| ≥1.09                    | 67    | 7   | 49  | 11  | 16.4 |          |      |      |      |             |      |      |      |             |
| CLDN-1                   |       |     |     |     |     |          |      |      |      |             |      |      |      |             |
| <1.06*                   | 87    | 19  | 56  | 12  | 13.8 | 0.491    | 1.00 | 1.27 | (0.60–2.69) | 0.74 | (0.24–2.25) | 0.806 | 100   | 1.35 | (0.63–2.89) | 0.78 | (0.25–2.40) |
| ≥1.06                     | 89    | 17  | 64  | 8   | 9.0  |          |      |      |      |             |      |      |      |             |
| <0.75**                  | 38    | 7   | 25  | 6   | 15.8 | 0.614    | 1.00 | 0.91 | (0.36–2.33) | 0.56 | (0.15–1.99) | 0.420 | 100   | 0.96 | (0.37–2.48) | 0.57 | (0.16–2.04) |
| ≥0.75                    | 138   | 29  | 95  | 14  | 10.1 |          |      |      |      |             |      |      |      |             |
| CLDN-2                   |       |     |     |     |     |          |      |      |      |             |      |      |      |             |
| <1.15*                   | 47    | 13  | 29  | 5   | 10.6 | 0.434    | 1.00 | 1.90 | (0.69–5.23) | 195  | (0.44–8.54) | 0.275 | 100   | 2.56 | (0.79–8.34) | 2.99 | (0.57–15.51) |
| ≥1.15                     | 48    | 8   | 34  | 6   | 12.5 |          |      |      |      |             |      |      |      |             |
| <1.12**                  | 16    | 6   | 8   | 2   | 12.5 | 0.241    | 1.00 | 2.75 | (0.82–9.15) | 1.80 | (0.29–10.90)| 0.269 | 100   | 2.97 | (0.84–10.44) | 2.04 | (0.32–12.94) |
| ≥1.12                    | 79    | 15  | 55  | 9   | 11.4 |          |      |      |      |             |      |      |      |             |
| CLDN-4                   |       |     |     |     |     |          |      |      |      |             |      |      |      |             |
| <0.85*                   | 86    | 17  | 59  | 10  | 11.6 | 0.963    | 1.00 | 0.91 | (0.43–1.92) | 0.84 | (0.33–2.89) | 0.930 | 100   | 0.93 | (0.43–2.01) | 1.04 | (0.35–3.12) |
| ≥0.85                     | 90    | 19  | 60  | 11  | 12.2 |          |      |      |      |             |      |      |      |             |
## Table 3 (continued)

| Expression levels of gene | Grade | Crude OR (95% CI) | Adj OR (95% CI) |
|---------------------------|-------|------------------|----------------|
|                           |       |                  |                |
|                           |       | P-value | I | II | III |       | I | II | III |       |
| <0.67**                   | 68    | 14 (206) | 47 (691) | 7 (10.3) | 0.866 | 1.00 | 0.97 | 1.27 | 0.740 |
| ≥0.67                     | 108   | 22 (204) | 72 (66.7) | 14 (13.0) | 0.687 | 0.687 |

The statistically significant finding was shown in italics (*P < 0.05*).

* N number, OR odds ratio, CI confidence interval, CTNN cortactin, RhoA ras homolog gene family member A, ROCK rho-associated kinase, CLDN claudin

| b | Logistic regression analysis was performed

** Adjusted for education status (illiterate/middle school/diploma/bachelor and higher) and waist circumference (< 90 cm/90 cm); c Age at menarche (< 13 year/13 year) and waist circumference (< 90 cm/90 cm); d Age at menarche (< 13 year/13 year) and number of pregnancy (0-1/2-3/≥4); e Age at first pregnancy (< 24 year/24 year) and body mass index (< 24.99 kg/m²/25-29.99 kg/m²/30 kg/m²); f Education status (illiterate/middle school/diploma/bachelor and higher) and body mass index (< 24.99 kg/m²/25-29.99 kg/m²/30 kg/m²); g Education status (illiterate/middle school/diploma/bachelor and higher) and number of pregnancy (0-1/2-3/≥4); h Age at menarche (< 13 year/13 year) and number of pregnancy (0-1/2-3/≥4).

** Chi-square test was performed

* Fold changes in the expression of studied genes were categorized based on median values

** Fold changes in the expression of studied genes were categorized based on the cut-off identified by Youden's index (ROC-based cut-off)

### Discussion

For the first time, the results of this molecular epidemiologic study provide insights about the inter-correlations of CTNN, CLDN, and ROCK in association with the clinical stages of primary BrCa. Moreover, the overexpression of CTNN, CLDN, and ROCK was positively correlated with the extent of tumor size, particularly in ER+ status. CLDN up-regulation was noted in advanced histologic tumor grade and lymph node involvement.

An earlier study indicated that protein and mRNA expression levels of ROCK were significantly correlated to nodal involvement. Similar to our findings, Lane et al. [28] showed that protein and mRNA expression levels of ROCK were significantly correlated to nodal involvement. Bottino et al. [31] reported ROCK overexpression in tumors with advanced clinical stages of primary BrCa. Moreover, the overexpression of ROCK was positively correlated to axillary lymph node involvement. Therefore, ROCK can promote the motility and adhesion of cancer cells in extravasation, thus might hold tumor dissemination possibility in lymphatic metastasis [53].

** A significant correlation was observed between ROCK and lymph node metasis.**

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For the first time, the results of this molecular epidemiologic study provide insights about the inter-correlations of CTNN, CLDN, and ROCK in association with the clinical stages of primary BrCa. Moreover, the overexpression of CTNN, CLDN, and ROCK was positively correlated with the extent of tumor size, particularly in ER+ status. CLDN up-regulation was noted in advanced histologic tumor grade and lymph node involvement.
Cortactin can potentially promote the polymerization and rearrangement of actin in the cellular cortex, which modulates actin cytoskeleton and related dynamics [13]. Findings of experimental animal models compared to MCF-7 tumor cells indicated that the mRNA level of CTTN might drive tumor cells to disseminate into lymphatic vessels and develop lymph node metastasis [58]. One study reported a significant correlation between the protein expression of cortactin and lymph node metastasis of breast tumors [22]. The present findings provide new evidence showing that inter-correlation of CTTN-ROCK could be significantly correlated to positive lymph node involvement. In addition, cortactin effects on actin remodeling and subsequent degradation of the extracellular matrix occur in cancerous dissemination might be mediated by GTPase RhoA activity [59, 60]. Studies for supporting CTTN-ROCK interaction are limited; however, Croucher et al. [60] indicated that cortactin is competent to induce RhoA transcription in a dose-dependent manner in the case of CTTN overexpression which is revealed in head and neck squamous cells. A considerable dose of cortactin may overcome the distorted link between cortactin and RhoA by negative co-effectors [60]. Also, cortactin-related RhoA activity is documented to show cellular proliferation in head and neck squamous cell carcinoma [60]. GTP-RhoA (active form) interacts with the C-terminal part of coiled-coil domain and activates ROCK which is the main downstream target of active RhoA [26]. Nevertheless, a few pieces of evidence represented a background for cortactin-related RhoA activation, regardless of metastatic features, this study suggests evidence indicating the association between CTTN and ROCK expressions in favor of ALNM development. The overexpression of CLDN-4 was observed in patients with lymphatic metastasis subgroup which is in agreement with previous reports of BrCa patients [19, 41]. The CLDN-4-dependent up-regulation of matrix metalloproteinase (MMP)-2 and MMP-9 and increased invasiveness might be another clue responsible for lymphatic metastasis [61].

Histologic grades and tumor size
This is the first study to indicate the higher mRNA expression levels of CTTN in significant correlations with larger tumor size and histologic grade II of primary BrCa. Similarly, the mRNA expression levels of CTTN was correlated to a larger tumor size in colon cancer [15] and non-small cell lung cancers [16]. Moreover, previous studies reported that protein expression of cortactin could be associated with advanced histologic grades and poor differentiation in the cancers of colon [20], pancreas [21], and lung [16]. Overexpressed CTTN might induce the expression of S-phase kinase-associated protein-2 (SKP-2) to promote the cellular proliferation of head and neck squamous cell carcinoma [60], which was associated with larger tumor size observed in BrCa patients [62, 63]. Besides, cortactin-related SKP-2 signaling and subsequent down-regulation of cyclin-dependent kinase inhibitors might promote rapid cellular proliferation to result in increased tumor size. On the other hand, Clark et al. [64, 65] explained that cortactin is a transcriptional regulator of MMPs. Insulin-like growth factors and epidermal growth factor are potential tumor growing effectors released by MMP’s proteolytic activities, and likely MMPs can enhance cellular proliferation [66]. The findings indicated CTTN expression associated with larger tumor size of BrCa could describe cortactin as an effective variable and call to question its role in advanced histologic grade of breast carcinoma for future studies.

The present findings showed that mRNA expression of CLDN-1 was positively correlated to larger tumor size in our population of BrCa. Similarly, in a previous study, the overexpression of CLDN-1 was reported in association with a larger tumor size [36]. CLDN-1 can interact with the epithelial to mesenchymal transition (EMT) related markers such as zinc finger protein SNAI-1 (Snail-1), zinc finger protein SNAI-2 (Slug), and zinc finger E-box binding homeobox-1 (Zeb-1) in human BrCa cell lines [44, 67, 68] and therefore can suppress E-cadherin which is an essential molecule incorporating into an active EMT. CLDN-1 might enhance Zeb-1 levels through phosphatidylinositol-3 kinase (PI3K)/protein kinase B (Akt) pathway and Wnt/β-catenin pathway to suppress E-cadherin-related EMT pathogenesis in colon cancer [69]. Furthermore, the overexpression of CLDN-1 might be connected to MMP-9/Notch signaling to describe cellular proliferation in colorectal cancer [70]. Notch signaling is another aspect that gives rise to the overexpression of cyclins (A, B, and D) which they incorporate in cell cycle progression [71]. Moreover, overexpressed CLDN-1 might improve MMP-2-mediated proliferation of vascular smooth muscle cells [72] which might enhance angiogenesis to integrate CLDN-1 to tumorigenesis. Also, the anti-apoptotic effect of CLDN-1 was indicated in tamoxifen-treated MCF-7 cell lines [73]. Our findings showed a positive correlation between CLDN-4 expression and larger tumor size. Likewise, the positive protein expression of CLDN-4 was reported in correlation with larger tumor size of BrCa of Egyptian women [43]. CLDN-4 integration into tight junctions might be reduced by phosphorylation— one of the post-translational modifications of CLDNs – and therefore leads to gate function loss in various cancer cell lines [74, 75]. It might be a possible mechanism for CLDN-4 overexpression in BrCa, as well. An
### Table 4  Odds ratios (ORs) and 95% confidence intervals (95%CI) of fold changes in the expression of CTTN, RhoA, ROCK, CLDN-1, CLDN-2, and CLDN-4 genes in association with molecular subtypes* of primary non-metastatic BrCa (no distant metastasis) (N = 206)

| Molecular subtype | Luminal B vs. Luminal A HER2 rich vs. Luminal A Triple-negative vs. Luminal A | Luminal A vs. Non-HER2 rich vs. Luminal A | Luminal B vs. Non-HER2 | HER2 rich vs. Non-Her2 Triple-negative vs. Non-triple negative |
|-------------------|---------------------------------|----------------------------------------|----------------------|---------------------------------------------------------------|
|                   | N                               | Crude Adj. Crude Adj. Crude Adj. Crude Adj. Crude Adj. |         |                                      |
|                   | 23/129                          | 13/129 9/129 9/198                    | 23/183              | 13/194                                                        | 9/198 |

#### CTTN

| Gene Status of expression | Crude OR (95%CI) | Adj. OR (95%CI) | Crude OR (95%CI) | Adj. OR (95%CI) | Crude OR (95%CI) | Adj. OR (95%CI) | Crude OR (95%CI) | Adj. OR (95%CI) | Crude OR (95%CI) | Adj. OR (95%CI) |
|---------------------------|-----------------|----------------|-----------------|----------------|-----------------|----------------|-----------------|----------------|-----------------|----------------|
| **High/Low**              |                 |                |                 |                |                 |                |                 |                |                 |                |
| High/Low**                |                 |                |                 |                |                 |                |                 |                |                 |                |
| RhoA                      |                 |                |                 |                |                 |                |                 |                |                 |                |
| High/Low*                 | 1.29 (0.52-3.22) | 1.25 (0.50-3.15) | 1.73 (0.53-5.58) | 2.16 (0.51-9.03) | 2.19 (0.52-9.16) | 0.83 (0.46-1.47) | 1.24 (0.50-3.02) | 1.66 (0.52-5.28) | 1.72 (0.53-5.39) | 0.91 (0.53-5.41) |
| High/Low**                | 1.08 (0.43-2.67) | 1.03 (0.41-2.60) | 1.73 (0.53-5.58) | 2.16 (0.51-9.03) | 2.19 (0.52-9.16) | 0.87 (0.49-1.50) | 1.03 (0.53-2.50) | 1.70 (0.53-5.39) | 1.70 (0.53-5.39) | 0.91 (0.53-5.41) |
| ROCK                      |                 |                |                 |                |                 |                |                 |                |                 |                |
| High/Low*                 | 1.01 (0.82-5.90) | 2.21 (0.81-5.99) | 1.02 (0.32-3.20) | 1.19 (0.37-3.82) | 4.38 (0.86-22.55) | 3.51 (0.00-11.11) | 1.97 (0.75-5.20) | 0.74 (0.27-2.61) | 3.68 (0.25-4.46) | 3.48 (0.70-17.26) |
| High/Low**                | 2.83 (1.7-7.43)  | 2.83 (1.7-7.43)  | 1.17 (0.36-3.82) | 1.25 (0.38-4.12) | 6.60 (1.33-33.82) | 0.71 (0.36-21.11) | 2.66 (1.02-6.93) | 1.32 (0.32-3.29) | 0.91 (0.28-2.91) | 0.58 (1.14-28.22) |
| CLDN-1                    |                 |                |                 |                |                 |                |                 |                |                 |                |
| High/Low*                 | 1.03 (0.37-2.22) | 0.93 (0.37-3.66) | 1.20 (0.38-3.80) | 1.00 (1.24-4.17) | 1.02 (0.24-4.30) | 0.99 (0.24-6.71) | 0.90 (0.38-3.62) | 1.00 (0.24-4.11) | 1.00 (0.24-4.11) | 1.00 (0.24-4.11) |
| High/Low**                | 1.62 (0.22-1.63) | 1.61 (0.22-1.64) | 1.67 (0.30-703)  | 1.48 (0.30-713)  | 0.44 (0.10-198)  | 0.66 (0.21-265)  | 0.64 (0.21-568)  | 0.72 (0.26-168)  | 0.47 (0.35-792)  | 0.47 (0.10-208)  |
| CLDN-2                    |                 |                |                 |                |                 |                |                 |                |                 |                |
| High/Low*                 | 1.55 (0.27-3.14) | 0.91 (0.26-3.03) | 0.34 (0.03-3.08) | 0.30 (0.03-3.08) | 0.30 (0.08-277)  | 1.27 (0.59-2.64) | 0.30 (0.08-277) | 0.30 (0.08-277) | 0.30 (0.08-277) | 0.30 (0.08-277) |
| High/Low**                | 1.13 (0.21-5.73) | 1.08 (0.21-5.62) | 1.34 (0.14-1214) | 1.28 (0.14-1177) | 0.66 (0.06-692)  | 0.81 (0.28-233)  | 0.81 (0.28-233) | 0.81 (0.28-233) | 0.81 (0.28-233) | 0.81 (0.28-233) |
| CLDN-4                    |                 |                |                 |                |                 |                |                 |                |                 |                |
| High/Low*                 | 1.01 (0.28-1.68) | 0.72 (0.25-1.76) | 1.79 (0.51-625)  | 1.83 (0.12-234)  | 0.75 (0.75-235)  | 0.74 (0.77-235)  | 0.74 (0.77-235) | 0.74 (0.77-235) | 0.74 (0.77-235) | 0.74 (0.77-235) |
| High/Low**                | 1.82 (0.23-1.38) | 0.57 (0.23-1.42) | 0.33 (0.05-1343) | 0.55 (0.07-136)  | 0.85 (0.07-314)  | 1.52 (0.86-572)  | 1.52 (0.86-572) | 1.52 (0.86-572) | 1.52 (0.86-572) | 1.52 (0.86-572) |
in vivo assay in nude mice was transplanted by CLDN-4-silenced MCF-7 cells indicated the regression in breast tumor size [42]. A meta-analysis confirmed the elevated protein levels of CLDN-4 in larger tumor size in gastric carcinoma [76]. Consistent with earlier studies [37, 40–43], CLDN-4 overexpression was associated with advanced histologic grade in present BrCa patients. The protein expression of CLDN-4 was positively correlated to Ki-67 labeling index among BrCa patients [37, 43], indicating that CLDN-4 might be contributed to proliferative pathways and cellular differentiation. Therefore, further studies in laboratory and epidemiologic outlooks are warranted to confirm further the association between CLDNs and advanced stages of BrCa.

**Hormone receptors**

The higher mRNA expression level of CTTN was found in luminal A than non-luminal A subtypes. Accordingly, a large-scale cohort of primary BrCa patients indicated CTTN overexpression in association with BrCa metastasis in ER+ samples [24]. Cortactin might increase the risk of breast adenocarcinoma metastasis to bone marrow mediated by hyaluronan/cluster of differentiation-44 (CD44) signaling in MCF-7 cell lines indicating that the expression level of CTTN can be positively regulated by CD44 [77]. Karamanou and colleagues [78] indicated less expression levels of CD44 and cortactin in ERα+ MCF-7 than levels observed in ERβ+MDA-MB-231 BrCa cells [78]. Magalhaes et al. [79] reported that the tyrosine phosphorylation of cortactin increased recruitment of Na+/H+ exchanger-1 (NHE-1) in MDA-MB-231 cell lines. The overactivation of NHE-1—a plasma membrane glycoprotein that controls intracellular pH—could result in an acidic extracellular microenvironment leading to breast tumor cell invasion and the development of metastasis [80]. It could collectively support our findings showing the overexpression of CTTN in luminal A subtype of BrCa, particularly when CTTN up-regulation was evident in pre-menopause BrCa patients.

The hyaluronan/CD44 signaling was also correlated to the activation of RhoA/ROCK pathway and subsequent the phosphorylation of NHE-1, leading to breast tumor cell invasion [81]. Consistent with Oviedo et al. [82] indicated that the presence of estradiol could result in increased protein and mRNA expression levels of RhoA in human umbilical vein endothelial cells; our findings represented an additional insight expressing the intercorrelation of RhoA-ROCK in association with ER positivity. We also demonstrated that the luminal B subtype was remarkable in tumors overexpressed ROCK, suggesting that high expression level of ROCK may be affected by HER2 expression of breast tumors. Exposure to physiological concentrations of 17β-estradiol in human
### Table 5  Univariate linear regression analysis (standardized $\beta$ coefficient) between the studied genes of tumors of study population of BrCa (N = 206)

|                | CTTN (n = 174) | RhoA (n = 205) | ROCK (n = 202) | CLDN-1 (n = 206) | CLDN-2 (n = 110) | CLDN-4 (n = 206) |
|----------------|----------------|----------------|----------------|------------------|------------------|------------------|
| **Total population** |                |                |                |                  |                  |                  |
| CTTN           | 1              |                |                |                  |                  |                  |
| RhoA           | 0.065 (0.395)* | 1              |                |                  |                  |                  |
| ROCK           | 0.170 (0.028)  | 0.246 (< 0.001)| 1              |                  |                  |                  |
| CLDN-1         | −0.036 (0.646) | −0.007 (0.917) | −0.062 (0.391) | 1                |                  |                  |
| CLDN-2         | 0.043 (0.686)  | −0.017 (0.866) | 0.237 (0.014)  | 0.034 (0.727)    | 1                |                  |
| CLDN-4         | 0.013 (0.868)  | 0.086 (0.228)  | −0.023 (0.752) | 0.411 (< 0.001)  | −0.102 (0.292)   | 1                |
| **ER- and PR± (n = 23)** |                |                |                |                  |                  |                  |
| CTTN           | 1              |                |                |                  |                  |                  |
| RhoA           | 0.182 (0.465)  |                |                |                  |                  |                  |
| ROCK           | 0.398 (0.091)  | 0.397 (0.067)  | 1              |                  |                  |                  |
| CLDN-1         | 0.198 (0.415)  | 0.009 (0.967)  | −0.049 (0.832) | 1                |                  |                  |
| CLDN-2         | 0.655 (0.029)  | −0.247 (0.465) | −0.285 (0.396) | −0.316 (0.344)   | 1                |                  |
| CLDN-4         | −0.027 (0.914) | 0.077 (0.748)  | 0.007 (0.975)  | 0.095 (0.691)    | −0.366 (0.299)   | 1                |
| **ER+ and PR± (n = 158)** |                |                |                |                  |                  |                  |
| CTTN           | 1              |                |                |                  |                  |                  |
| RhoA           | 0.074 (0.404)  | 1              |                |                  |                  |                  |
| ROCK           | 0.128 (0.153)  | 0.280 (0.001)  | 1              |                  |                  |                  |
| CLDN-1         | −0.065 (0.469) | −0.001 (0.994) | −0.066 (0.433) | 1                |                  |                  |
| CLDN-2         | 0.033 (0.786)  | −0.005 (0.965) | 0.267 (0.016)  | 0.020 (0.858)    | 1                |                  |
| CLDN-4         | 0.016 (0.859)  | 0.086 (0.301)  | −0.015 (0.862) | 0.451 (< 0.001)  | −0.119 (0.284)   | 1                |
| **ALNM– (n = 64)** |                |                |                |                  |                  |                  |
| CTTN           | 1              |                |                |                  |                  |                  |
| RhoA           | −0.056 (0.705) | 1              |                |                  |                  |                  |
| ROCK           | −0.049 (0.741) | 0.233 (0.083)  | 1              |                  |                  |                  |
| CLDN-1         | 0.070 (0.638)  | 0.011 (0.932)  | −0.166 (0.217) | 1                |                  |                  |
| CLDN-2         | −0.041 (0.847) | 0.166 (0.382)  | 0.733 (< 0.001)| −0.045 (0.810)   | 1                |                  |
| CLDN-4         | 0.022 (0.882)  | 0.103 (0.446)  | −0.194 (0.151) | 0.522 (< 0.001)  | −0.131 (0.490)   | 1                |
| **ALNM+ (n = 120)** |                |                |                |                  |                  |                  |
| CTTN           | 1              |                |                |                  |                  |                  |
| RhoA           | 0.129 (0.204)  | 1              |                |                  |                  |                  |
| ROCK           | 0.226 (0.027)  | 0.311 (0.001)  | 1              |                  |                  |                  |
| CLDN-1         | −0.079 (0.448) | −0.010 (0.921) | 0.040 (0.681)  | 1                |                  |                  |
| CLDN-2         | 0.121 (0.384)  | −0.160 (0.219) | −0.098 (0.443) | 0.004 (0.976)    | 1                |                  |
| CLDN-4         | 0.030 (0.769)  | 0.068 (0.479)  | 0.029 (0.763)  | 0.377 (< 0.001)  | −0.028 (0.826)   | 1                |
| **Stage I (n = 55)** |                |                |                |                  |                  |                  |
| CTTN           | 1              |                |                |                  |                  |                  |
| RhoA           | 0.060 (0.710)  | 1              |                |                  |                  |                  |
| ROCK           | 0.519 (0.001)  | 0.252 (0.077)  | 1              |                  |                  |                  |
| CLDN-1         | −0.025 (0.877) | −0.060 (0.674) | −0.115 (0.422) | 1                |                  |                  |
| CLDN-2         | −0.064 (0.766) | 0.204 (0.298)  | 0.741 (< 0.001)| −0.049 (0.800)   | 1                |                  |
| CLDN-4         | 0.232 (0.144)  | 0.039 (0.758)  | 0.002 (0.990)  | 0.389 (0.004)    | −0.136 (0.480)   | 1                |
| **Stages II-III (n = 126)** |                |                |                |                  |                  |                  |
| CTTN           | 1              |                |                |                  |                  |                  |
| RhoA           | 0.098 (0.329)  | 1              |                |                  |                  |                  |
| ROCK           | 0.069 (0.496)  | 0.308 (0.001)  | 1              |                  |                  |                  |
| CLDN-1         | 0.002 (0.980)  | 0.022 (0.818)  | 0.002 (0.987)  | 1                |                  |                  |
| CLDN-2         | 0.130 (0.359)  | −0.163 (0.213) | −0.130 (0.324) | 0.013 (0.923)    | 1                |                  |
| CLDN-4         | −0.052 (0.609) | 0.085 (0.375)  | −0.019 (0.842) | 0.429 (< 0.001)  | −0.014 (0.917)   | 1                |
umbilical vein endothelial cells resulted in the interaction between ERα and Gα13 (heterotrimeric G protein) to induce RhoA/ROCK activity [83]. The RhoA/ROCK signaling pathway could be activated by nuclear factor erythroid 2-related factor 2 (NRF-2) [84]. The treatment with estradiol could activate PI3K/glycogen synthase kinase-3 beta pathway to increase the activity of NRF-2 in MCF-7 cells [85]. A meta-analysis of clinical studies reported that NRF2 overexpression was associated with a worse clinical outcomes of BrCa patients [86]. Another mechanism explains that in the presence of 17β-estradiol, ER may interact with c-Src to activate PI3K/Akt/RhoA pathway in human T47-D BrCa cells [87]. However, still, laboratory studies are insufficient to prove RhoA/ROCK bi-functionalities associated with the presence of certain molecular subtypes of BrCa.

ROCK may act as an upstream regulator to control CLDNs transcription [33]. The present study revealed a positive inter-correlation between ROCK-CLDN-2 in the case of ER positivity of tumors. In addition, the inter-correlation of CLDN-1-CLDN-4 was found abundant among the present ER+ tumors. CLDN-4 up-regulation was seen in the estrogen-related tissues such as the breast and ovaries [88, 89]. Blanchard et al. [44] reported protein expression of CLDN-1 in positive association with CLDN-4 in basal-like and non-basal breast tumors. Akimoto et al. [90] represented a positive correlation between the activity of estrogen-dependent G protein-coupled receptor 30/Akt-related pathway and CLDN-1 expression in cervical adenocarcinoma cells [90]. The contradictory results from studies which addressed CLDNs expression concerning molecular subtypes [35–38, 40, 41, 43, 44], present findings could add an insight focusing on the association of CLDN-1-CLDN-4 and ROCK-CLDN-2 connected to breast tumors characterized by hormone receptor-positive.

The present significant correlation between RhoA and ROCK among breast tumors would give rise to the importance of ROCK transcription in accounting the present variation of RhoA expression. Several previous studies have considered ROCK expression as a reliable reference to determine the accuracy of RhoA expression in Her2-rich breast tissues [30], cervical cancer [54], and bladder cancer [57]. Consistent with the present findings, the protein expression of CLDN-1 was previously suggested as a biomarker to determine the accuracy of CLDN-4 expression in BrCa [44].

This study had some limitations. First, the sample size was small for subgroup analysis. Second, pathologic data were collected prospectively after surgery; therefore, data were not available for some cases. Third, the correction for multiplicity testing was a statistical approach warranted for future studies to provide better clusters of genes interdependently associated with the invasive pathological features of BrCa. Fourth, this study could not provide information about diagnostic accuracy based on the area under the curve of ROC, sensitivity, specificity, likelihood ratio, or other statistical parameters to interpret the diagnostic accuracy of pro-metastatic genes in detection or predicting BrCa features. Thereafter, we could not precisely address the accuracy of biomarkers to predict other disease diagnoses. The present findings provided some new evidence; further studies need to determine their prognostic impact on pathological characteristics in BrCa using a gold standard as reference. Accordingly, evidence should support pro-metastatic genes in association with advanced features before conducting any assessment for diagnostic accuracy of biomarkers. Microarray techniques could be suggested for future studies to assess a broader number of genes encompasses several pathways such as genes involved in controlling antioxidant defense system in association with RhoA transcription.

**Conclusions**

In summary, findings could suggest the binary settings of pro-metastatic genes, including CTTN-ROCK and RhoA-ROCK in association with a breast tumor diagnosed with infiltration into axillary lymph nodes which is representative of local breast metastasis. Findings put emphasize on ROCK transcription as a contributor to ALNM of IDC -BrCa. The present findings indicated the overexpression of CTTN, CLDN-1, and CLDN-4 in association with advanced stages of primary non-metastatic BrCa, which is highly evident in ER+ status of breast tumors. Endocrine therapy might correlate with ER/PR related pro-metastatic genes that need further implications by future studies. However, further experimental studies are necessary to reveal mechanisms underlying gene-to-gene interactions in association with the molecular events representative of metastatic hallmarks of BrCa.
**Supplementary information**

The online version contains supplementary material available at https://doi.org/10.1186/s12935-020-01708-8.

**Additional file 1: Fig S1.** The overexpression of pro-metastatic genes were associated with clinicopathologic features of breast cancer. CLDN claudin, CTTN cortactin, HGPRT hypoxanthine–guanine phosphoribosyltransferase, NTC non-template control, Rh0A ras homolog gene family member A, ROCK rho-associated kinase, N normal, T tumor.

**Additional file 2: Table S1.** Primer sequences used for qRT-PCR amplification.

**Additional file 3: Table S2.** General characteristics of patients with invasive breast cancer (N = 206).

**Additional file 4: Table S3.** Associations between lymphatic invasion and the involvement of axillary lymph node metastasis (ALNM).

**Abbreviations**

ALNM: Axillary lymph node metastasis; ANCOVA: One-way analysis of covariance; BrCa: Breast cancer; BMI: Body mass index; CLDN: Claudin; CTTN: Cortactin; CI: Confidence interval; ER: Estrogen receptor; HER2: Human epidermal growth factor receptor-2; MMP: Matrix metalloproteinase; PR: Progesterone receptor; qRT-PCR: Quantitative real-time reverse transcriptase-polymerase chain reaction; OR: Odds ratio; RhoA: Ras homolog gene family member-A; ROCK: Rho-associated kinase.

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**Authors’ contributions**

SM. Supervision; Investigation; Resources; Writing—review & editing; MF. Formal analysis; Investigation; Methodology; Software; Writing—original draft; VM. Supervision; Conceptualization; Methodology; GT. Investigation; Resources; AF. Conceptualization; Methodology; Software; Writing—review & editing. This article was extracted using a dataset out of a Ph.D. thesis titled “The association between dietary pattern and expression levels of HIF-Tα, ROCK, and CTTN genes in breast cancer patients”, registered at SUMS, Shiraz, Iran (Registration no.: 94-01-84-10880) and TUMS, Tabriz, Iran (Registration no.: S/D/962552).

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**Availability of data and materials**

Data cannot be shared publicly due to legal restrictions imposed by the Ethics Committee of Tabriz University of Medical Sciences. Data are available by the Ethics Committee of Tabriz University of Medical Sciences (research-vice@tbzmed.ac.ir), for researchers who meet the criteria for access to confidential data.

**Ethics approval and consent to participate**

Before interviewing, written consent was obtained from all participants. The study was designed and carried out according to the Ethical Guidelines for Observational Studies [81]. The full study protocol was reviewed and approved by the ethics committee of both Tabriz University of Medical Sciences (Ethics no: IR.TBZMED.REC.1396.438) and Shiraz University of Medical Sciences (Ethics no: IR.SUMS.REC.1395.S378). This report was prepared in compliance with the STROBE statements [92].

**Consent for publication**

Not applicable.

**Competing interests**

The authors have declared that no competing interests exist.

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