SPANDIDOS PUBLICATIONS

ONCOLOGY LETTERS 19: 52-60, 2020

RAB6C is an independent prognostic factor of estrogen receptor-positive/progesterone receptor-negative breast cancer

HELENA FOHLIN[1,2], TOVE BEKKHUS[2], JOSEFINE SANDSTRÖM[2], TOMMY FORNANDER[3], BO NORDENSKJÖLD[2], JOHN CARSTENSEN[4] and OLLE STÅL[2]

[1]Regional Cancer Center of Southeast Sweden and Department of Clinical and Experimental Medicine, Linköping University, SE-581 85 Linköping; [2]Department of Clinical and Experimental Medicine, and Department of Oncology, Linköping University, SE-581 83 Linköping; [3]Department of Oncology, Karolinska University Hospital, Karolinska Institute, SE-171 77 Stockholm; [4]Department of Medical and Health Sciences, Division of Health and Society, Linköping University, SE-581-83 Linköping, Sweden

Received March 29, 2019; Accepted September 30, 2019

DOI: 10.3892/ol.2019.11109

Abstract. The majority of breast cancer tumors are estrogen receptor-positive (ER^+) and can be treated with endocrine therapy. However, certain patients may exhibit a good prognosis without systemic treatment. The aim of the present study was to identify novel prognostic factors for patients with ER^+ breast cancer tumors using gene copy data, and to investigate if these factors have prognostic value in subgroups categorized by progesterone receptor status (PR). Public data, including the whole genome gene copy data of 199 systemically untreated patients with ER^+ tumors, were utilized in the present study. To assess prognostic value, patients were divided into two groups using the median gene copy number as a cut-off for the SNPs that were the most variable. One SNP was identified, which indicated that the Ras-related protein Rab-6C (*RAB6C*) gene may exhibit prognostic significance. Therefore, RAB6C protein expression was subsequently investigated in a second independent cohort, consisting of 469 systematically untreated patients (of which 310 were ER^+) who received long term follow-up. In the public data set, a distant recurrence risk reduction of 55% was determined for copy numbers above the median value of *RAB6C* compared with numbers below [multivariable adjusted hazard ratio (HR), 0.45; 95% CI 0.28-0.72; P=0.001)]. It was also more pronounced in the ER^+/PR^- subgroup (HR, 0.15; 95% CI, 0.05-0.46; P=0.001). In the second cohort, patients of the ER^+/PR^- subgroup who exhibited high RAB6C expression had a reduced distant recurrence risk (HR, 0.17; 95% CI, 0.05-0.60; P=0.006). However, this was not identified among ER^+/PR^+ tumors (HR, 1.31; 95% CI, 0.69-2.48; P=0.41). The results of the present study indicated that RAB6C serves as an independent prognostic factor of distant recurrence risk in systemically untreated patients with an ER^+/PR^- tumor.

Correspondence to: Dr Helena Fohlin, Regional Cancer Center of Southeast Sweden and Department of Clinical and Experimental Medicine, Linköping University, Brigadgatan 21, SE-581 85 Linköping, Sweden
E-mail: helena.fohlin@regionostergotland.se

Abbreviations: CI, confidence interval; ER, Estrogen receptor; HER2, human epidermal growth factor receptor 2; HR, hazard ratio; IHC, immunohistochemistry; PR, progesterone receptor; RAB6C, Ras-related protein Rab-6C; SNP, single nucleotide polymorphism; TMA, tissue microarray

Key words: breast neoplasm, Ras-related protein Rab-6C, gene copy number, prognostic, estrogen receptor, progesterone receptor

Introduction

Breast cancer is a heterogeneous disease that can be divided into different subtypes based on pathological markers. One of the most important markers is the estrogen receptor (ER). Estrogen receptor-positive (ER^+) and estrogen receptor-negative (ER^-) tumors differ in their recurrence patterns. Patients with ER^- tumors exhibit higher recurrence risks over the first 5 years following diagnosis. Thereafter, the risk of recurrence decreases rapidly. However, patients with ER^+ tumors experience late recurrences more frequently. The biological differences between ER^+ and ER^- tumors are also evident. Several studies have revealed that different chromosomal and gene expressional patterns are present in patients with different ER statuses (1-3).

The majority of breast cancer tumors (75-80%) are ER^+ and of these, ~75% are also progesterone receptor-positive (PR^+) (4,5). ER^+/PR^- breast cancer is associated with a more aggressive phenotype (6), larger tumors, axillary lymph node metastases and higher S-phase fractions compared with ER^+/PR^+ tumors (7). Differences between these subgroups have also been observed at the DNA level. ER^+/PR^- tumors exhibit a more unstable genetic profile and possess twice as many copy number gains or losses as ER^+/PR^+ tumors (8). Thus, separate analyses for subgroups based on hormone receptor status is appropriate.

The aim of the current study was to identify new prognostic factors for ER^+ breast cancer via gene copy number analysis and to investigate if these factors had prognostic value in subgroups created based on PR status.

The present study identified the gene *RAB6C*, located on chromosome 2q21.1, which encodes a 254 amino acid protein. It was determined to be a prognostic marker following analysis and was subsequently investigated for prognostic value in a second independent data set from a study with systemically untreated patients. To the best of our knowledge, this is the first study to determine the prognostic value of RAB6C in clinical breast cancer tissue. However, experimental studies have demonstrated that RAB6C interacts with p53, which is frequently mutated in breast cancer (9-12). RAB6C has also been revealed to inhibit cell proliferation, invasion and metastases, leading to the hypothesis that it may function as a tumor suppressor (13).

Materials and methods

Patients of the public dataset. The current study utilized a public data set obtained from the tumor bank of the Erasmus Medical Center, which included 313 patients with breast cancer. Data is available at the NCBI GEO website (accession no. 10099) and includes information pertaining to gene copy number, clinical data and information on distant recurrences. The patients were treated between 1980 and 1995, and all were determined to be lymph node negative. Patients also did not receive any adjuvant systemic therapy. Gene copy number was previously analyzed using the Affymetrix GeneChip Human Mapping 100K Array. Additionally, Affymetrix Chromosome Copy Number Tool 3.0 software was used to generate a value representing the copy number of each probe set. The previously determined data was determined by Zhang *et al* (3). From this cohort, the current study selected ER^+ tumors (n=199) for further analyses.

Hormone receptors and grade of the public dataset. Protein levels of ER and PR were measured using a ligand binding assay, an enzyme immunoassay or immunohistochemistry (IHC) for a selection of tumors. The cut-off value for the classification of patients as positive or negative for ER and PR was 10 fmol/mg protein or 10% positive tumor cells (14). Grade was assessed by regional pathologists and reflected the current practice of clinicians over the years that the tumor samples were collected (3).

Patients of the independent cohort. To investigate the prognostic value of RAB6C at the protein level and to analyze a cohort comparable with GSE10099, systemically untreated patients were selected from two randomized studies conducted by the Stockholm breast cancer group between 1976 and 1990 (15,16). One study included postmenopausal 'low-risk' patients (tumor size ≤30 mm and lymph node-negative) and the other included premenopausal 'high-risk' patients (tumor size >30 mm and/or lymph node positive). After selecting systemically untreated patients, the cohort of the current study consisted of 1,150 patients, where tissue microarrays were available for 548 tumors. Of these, RAB6C expression could be evaluated in a total of 469 patients (Fig. 1).

Hormone receptors, HER2 status, Grade and RAB6C of the independent cohort. ER, PR and HER2 data were collected from previous studies. For postmenopausal patients, ER and PR status was assessed retrospectively via IHC using the Ventana® automated slide stainer (Ventana Medical Systems, S.A.). CONFIRM™ mouse anti-ER primary monoclonal antibodies (clone 6F11) and CONFIRM™ mouse anti-PR antibodies (clone 16) were obtained from Ventana Medical Systems. The cut-off level of positively stained tumor cell nuclei was set to 10% (17). HER2 was analyzed via IHC as previously described (17). For premenopausal patients, the ER and PR status that was determined during clinical routine practice was assessed with a cut-off level of 0.05 fmol/μg DNA (18). HER2 was analyzed via IHC using the same antibody as for postmenopausal patients. Grade was analyzed retrospectively in both cohorts using the same investigator for all tumor samples within each.

The protein expression of RAB6C was analyzed via IHC and the staining pattern was evaluated independently by three investigators (JS, TB and HF). Polyclonal rabbit antibodies (cat. no. ab200396; Abcam) were used. The intensity of RAB6C in the nucleus was analyzed and scored as 0, 1, 2 or 3. If the nuclei exhibited an intensity ≥2, the tumor was considered to highly express RAB6C ($RAB6C^+$). Otherwise, it was considered to exhibit a low RAB6C expression ($RAB6C^-$). Fig. S1 presents examples of tumors that were graded as $RAB6C^+$ and $RAB6C^-$, respectively. RAB6C expression in the cytoplasm was also evaluated and scored as 0, 1, 2 or 3.

Statistical analysis. The interquartile range was calculated for each SNP and the 20 most varied were selected and analyzed separately. For each SNP, the patients were divided into two groups based on their gene copy number, with the median value as a cut-off. To compare the association between RAB6C and clinical characteristics, the Pearson χ^2 test was utilized.

Cumulative distant-recurrence risk was estimated using the Kaplan-Meier method. In the public data set, distant recurrence was calculated as previously described by Zhang *et al* (3). In the independent cohort, the end-point was defined as the first distant recurrence from the patient's primary breast tumor as described by Rutqvist and Johansson (15,16). In this cohort, 3 patients died from breast cancer, but no date of distant recurrence was recorded. For these patients, the date of death was used as date of distant recurrence. Patients were censored at the last follow-up or at death due to causes other than breast cancer. Hazard ratios (HRs) and 95% confidence intervals (CIs) were estimated using Cox's proportional hazards model. P-values were obtained from two-sided Wald tests and the patients were followed up until 10 years after diagnosis. The microarray data was processed using R 2.14.1 and the statistical analyses were performed with Stata/SE 13.1 software.

Results

Public data set results. The 20 SNPs that varied the most between patients were mapped as *MECT1, UBN1* (3 SNPs), *SKI, GALNT1, FLJ35424, FCGR1A, WIG1, RAB6C, MUM1L1*, 7p22.3, *POU5FLC20, ZNF195, LOC200030, LOC122618, ODF1* and *OSR2* (3 SNPs; Table SI). When analyzing the distant recurrence risk of each SNP after applying Bonferroni's correction, only *RAB6C* had a statistically significant impact on prognostic value. Furthermore, since previous studies indicated that RAB6C might be a favorable prognostic and

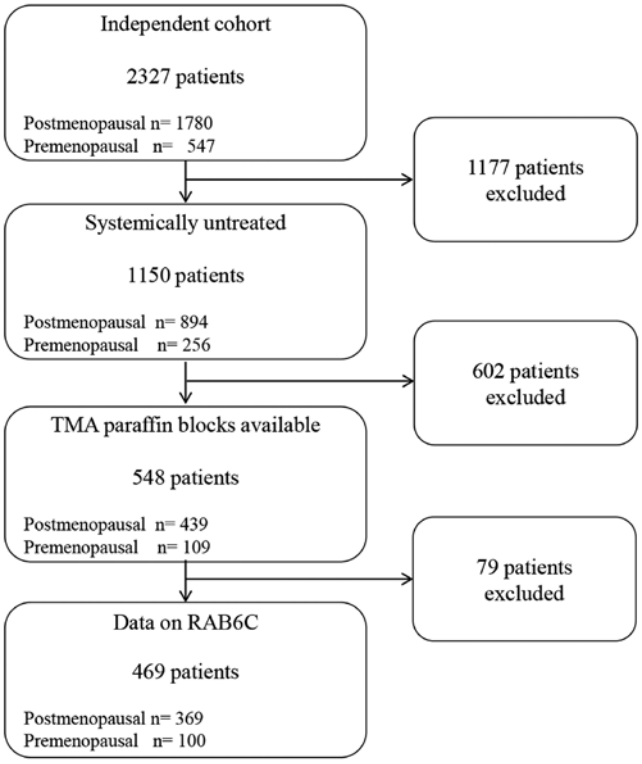

Figure 1. Consort diagram of the independent cohort. RAB6C, Ras-related protein Rab-6C; TMA, tissue microarray.

predictive factor, this gene was selected for further analysis (11,13,19,20).

The second most significant P-value was obtained for the *MUM1L1* gene (HR, 0.55; 95% CI, 0.35-0.86; P=0.01). The protein that the *MUM1L1* gene encodes has been previously revealed to be highly expressed in several types of cancer. According to the protein atlas, the expression of *MUM1L1* is not detectable in normal breast tissue and is expressed in low levels in breast cancer tissue (21). However, due to a non-significant P-value after Bonferroni's correction, no further analysis was performed on *MUM1L1* in the current study.

The patients that were subdivided into groups according to their tumor copy number being above or below the median value of 3.6 were denoted as the *RAB6C*$^+$ and *RAB6C*$^-$ groups, respectively. The HR of the patients that exhibited a *RAB6C*$^+$ tumor compared with those that exhibited a *RAB6C*$^-$ tumor was 0.44 (95% CI, 0.28-0.71; P=0.001; Fig. 2A). The prognostic value of *RAB6C* was determined by performing a multivariable analysis with adjustments for age, tumor stage, grade and PR. Of these factors, *RAB6C* had the strongest prognostic value (HR, 0.45; 95% CI, 0.28-0.72; P=0.001; Tables I and SII).

The patients were further divided into two subgroups based on PR status. In both subgroups, *RAB6C* had a favorable prognostic value, which was more evident among ER$^+$/PR$^-$ patients. In these patients, there was a 77% risk reduction (HR, 0.23; 95% CI, 0.08-0.61; P=0.003) in tumors that were *RAB6C*$^+$ compared with those that were *RAB6C*$^-$ (Fig. 2B). For ER$^+$/PR$^+$ tumors, the risk reduction was 45% (HR, 0.55; 95% CI, 0.32-0.96; P=0.03; Fig. 2C). Based on PR status, two separate multivariable analyses were performed. *RAB6C*$^+$ revealed a statistically significant improvement in the prognosis of both subgroups. However, a stronger independent prognostic value was identified among ER$^+$/PR$^-$ tumors (HR, 0.15; 95% CI, 0.05-0.46 vs. HR, 0.55; 95% CI, 0.32-0.95; Tables I and SIII).

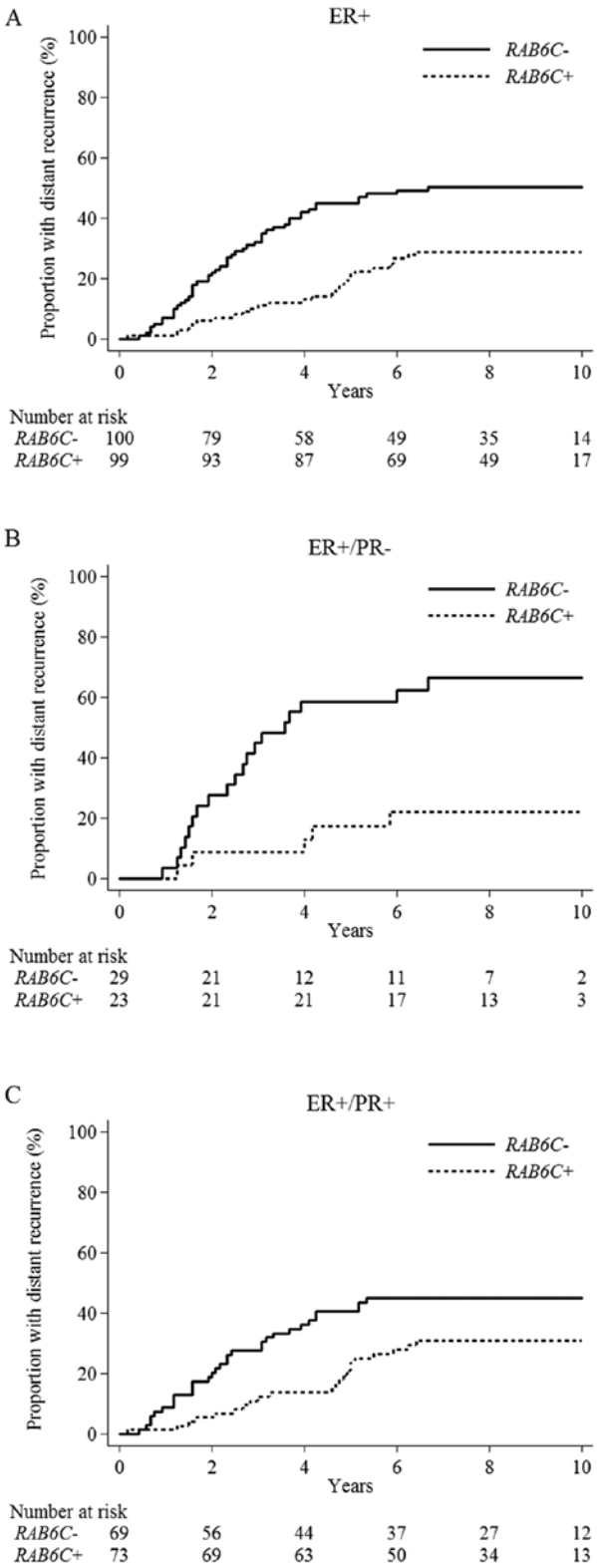

Figure 2. Cumulative distant recurrence risk in relation to the gene copy number of *RAB6C* in patients of the public dataset. (A) ER$^+$. HR, 0.44 (95% CI, 0.28-0.71; P=0.001). (B) ER$^+$/PR$^-$. HR, 0.23 (95% CI, 0.08-0.61; P=0.003). (C) ER$^+$/PR$^+$. HR, 0.55 (95% CI, 0.32-0.96; P=0.03). ER, estrogen receptor; HR, hazard ratio; PR progesterone receptor; RAB6C, Ras-related protein Rab-6C.

Table I. Distant recurrence rate in the data set GSE10099 for *RAB6C*$^+$ compared with *RAB6C*$^-$ stratified by hormone receptor status.

	Number of patients/events		Univariable HR (95% CI)		Multivariable[a] HR (95% CI)	
Hormone receptor status	*RAB6C*$^+$	*RAB6C*$^-$	*RAB6C*$^+$ vs. *RAB6C*$^-$	P-value	*RAB6C*$^+$ vs. *RAB6C*$^-$	P-value
ER$^+$	99/28	100/50	0.44 (0.28-0.71)	0.001	0.45 (0.28-0.72)	0.001
ER$^+$/PR$^+$	73/22	69/31	0.55 (0.32-0.96)	0.034	0.55 (0.32-0.95)	0.033
ER$^+$/PR$^-$	23/5	29/19	0.23 (0.08-0.61)	0.003	0.15 (0.05-0.46)	0.001

[a]Multivariable analysis for ER$^+$ tumors adjusted for age, tumor stage, grade and PR. The multivariable analysis confined to the subgroups ER$^+$/PR$^+$ and ER$^+$/PR$^-$ adjusted for age, tumor stage and grade. *RAB6C*$^-$ was the referent (with HR=1) in each subgroup analysis. ER, estrogen receptor; HR, hazard ratio; PR, progesterone receptor; RAB6C, Ras-related protein Rab-6C.

Table II. Patient characteristics for the data set GSE10099, stratified by hormone receptor status.

	ER$^+$, n (%)			ER$^+$/PR$^+$, n (%)			ER$^+$/PR$^-$, n (%)		
Characteristics	*RAB6C*$^-$	*RAB6C*$^+$	P-value	*RAB6C*$^-$	*RAB6C*$^+$	P-value	*RAB6C*$^-$	*RAB6C*$^+$	P-value
Total no. of patients	100	99		69	73		29	23	
Age, years									
≤50	46 (46)	38 (38)	0.28	37 (54)	30 (41)	0.14	9 (31)	7 (30)	0.96
>50	54 (54)	61 (62)		32 (46)	43 (59)		20 (69)	16 (70)	
Tumour stage									
T1	49 (49)	55 (56)	0.36	32 (46)	41 (56)	0.24	16 (55)	11 (48)	0.60
T2-T4	51 (51)	44 (44)		37 (54)	32 (44)		13 (45)	12 (52)	
PR status									
Negative	29 (30)	23 (24)	0.38						
Positive	69 (70)	73 (76)							
Unknown	2	3							
Grade									
I-II	17 (26)	22 (32)	0.40	14 (35)	11 (23)	0.23	3 (13)	9 (50)	<0.01
III	49 (74)	46 (68)		26 (65)	36 (77)		21 (88)	9 (50)	
Unknown	34	31		29	26		5	5	

ER, estrogen receptor; PR, progesterone receptor; RAB6C, Ras-related protein Rab-6C.

A higher gene copy number of *RAB6C* was associated with favorable prognostic factors. *RAB6C*$^+$ tumors were more frequently of T1 size (56 vs. 49%), PR$^+$ (76 vs. 70%) or of grade I or II (32 vs. 26%) when compared with *RAB6C*$^-$ tumors. However, none of these associations were statistically significant. In the ER$^+$/PR$^-$ subgroup, *RAB6C*$^+$ tumors were more commonly grade I or II (P=0.008; Table II).

Independent cohort results. The prognostic value of RAB6C based on protein expression was tested in two different cohorts that included premenopausal 'high-risk' patients and post-menopausal 'low-risk' patients, respectively. Since statistical analysis revealed similar results in both cohorts, they were merged into a single cohort to increase the statistical power. Cytoplasmic RAB6C expressions were observed in almost all cases (96%) with staining intensities of 2 or 3 in 71%. The results of nuclear staining were more varied, with scores of 0, 1, 2 and 3 in 34, 23, 25 and 19% of cases, respectively. The statistical analysis of cytoplasmic RAB6C expression did not demonstrate any prognostic value and as such, all results included in the current study were based on the nuclear expression of RAB6C.

The dataset contained both ER$^+$ and ER$^-$ tumors, which revealed a strong positive correlation between RAB6C and ER status (P=0.001). However, a negative correlation with borderline significance was observed between RAB6C and HER2 (P=0.057). RAB6C$^+$ tumors were also frequently of a lower grade than RAB6C$^-$ tumors (P<0.001; Table III). The current study subsequently analyzed the association between RAB6C and the three individual components of grading

Table III. Patient characteristics for the independent cohort.

	All patients, n (%)			ER$^+$, n (%)		
Characteristics	RAB6C$^-$	RAB6C$^+$	P-value	RAB6C$^-$	RAB6C$^+$	P-value
Total no. of patients	265	204		157	153	
Age, years						
≤50	61 (23)	44 (22)	0.71	38 (24)	34 (22)	0.68
>50	204 (77)	160 (78)		119 (76)	119 (78)	
Tumour size, mm						
<30	218 (85)	181 (90)	0.14	134 (87)	137 (90)	0.39
≥30	37 (15)	20 (10)		20 (13)	15 (10)	
Unknown	10	3		3	1	
Lymph node status						
N0	214 (81)	169 (83)	0.56	124 (79)	127 (83)	0.37
N$^+$	51 (19)	35 (17)		33 (21)	26 (17)	
ER status						
Negative	68 (30)	30 (16)	0.001			
Positive	157 (70)	153 (84)				
Unknown	40	21				
PR status						
Negative	111 (51)	78 (45)	0.24	50 (35)	51 (37)	0.76
Positive	105 (49)	94 (55)		92 (65)	87 (63)	
Unknown	49	32		15	15	
HER2						
Negative	197 (84)	166 (90)	0.057	134 (91)	138 (96)	0.07
Positive	38 (16)	18 (10)		14 (9)	6 (4)	
Unknown	30	20		9	9	
NHG						
I	38 (17)	50 (28)	<0.001	32 (23)	45 (32)	0.10
II	119 (52)	106 (60)		86 (61)	81 (58)	
III	73 (32)	21 (12)		23 (16)	14 (10)	
Unknown	35	27		16	13	

ER, estrogen receptor; NHG, Nottingham histological grade; PR, progesterone receptor; RAB6C, Ras-related protein Rab-6C.

(pleomorphism, tubule formation and mitosis). The results revealed a negative correlation between RAB6C positivity and each of the components (P<0.001). Among the ER$^+$ tumors, no statistically significant associations between RAB6C and the prognostic factors presented in Table III were detected, but HER2 was negativity associated with RAB6C$^+$ tumors (P=0.07). ER$^+$/PR$^-$ tumors with high RAB6C levels were frequently of a lower grade (P=0.016; Table IV).

Since the analyses performed using the public dataset were based on ER$^+$ tumors, the independent cohort was divided into two groups based on ER status. Among these subgroups, no statistically significant differences were determined in distant recurrence rates between RAB6C$^+$ and RAB6C$^-$ cases, independently of ER status. For patients with ER$^+$ tumors, the HR was 0.77 (95% CI, 0.48-1.22; P=0.27; Fig. 3A) and for ER$^-$tumors, the HR was 1.30 (95 % CI, 0.66-2.56; P=0.45).

For patients with ER$^+$/PR$^-$ tumors, a decreased distant recurrence rate was observed in RAB6C$^+$ compared with RAB6C$^-$ cases (HR, 0.24; 95% CI, 0.09-0.66; P=0.005; Fig. 3B). However, no statistically significant differences were determined in patients with ER$^+$/PR$^+$ tumors (HR, 1.20; 95% CI, 0.66-2.19; P=0.55; Fig. 3C). The separate HRs for the 'low-risk' and 'high-risk' cohorts with ER$^+$/PR$^-$ tumors were 0.22 (95% CI, 0.06-0.79) and 0.21 (95% CI, 0.041-1.10), respectively. For those with ER$^+$/PR$^+$ tumors, the HRs were 1.56 (95% CI, 0.72-3.41) and 1.21 (95% CI, 0.44-3.31), respectively (Fig. S2).

When adjusting for age, tumor size, lymph node status, HER2, grade and PR in the multivariable analysis of distant recurrence risk in patients with ER$^+$ tumors, RAB6C retained its prognostic importance, which was stronger in patients with ER$^+$/PR$^-$ tumors as indicated by a statistically significant interaction between PR and RAB6C (Table SIV). Stratifying the results of the multivariable analysis according to PR status revealed that the distant recurrence rate ratio for high vs. low RAB6C was markedly reduced among patients with ER$^+$/PR$^-$ tumors (HR, 0.17; 95% CI, 0.05-0.60; P=0.006), but not in

Table IV. Patient characteristics for the independent cohort stratified by hormone receptor status.

	ER^+/PR^+, n (%)			ER^+/PR^-, n (%)		
Characteristics	$RAB6C^-$	$RAB6C^+$	P-value	$RAB6C^-$	$RAB6C^+$	P-value
Total no. of patients	92	87		50	51	
Age, years						
≤50	22 (24)	14 (16)	0.19	9 (18)	11 (22)	0.65
>50	70 (76)	73 (84)		41 (82)	40 (78)	
Tumour size, mm						
<30	79 (87)	79 (91)	0.40	41 (85)	46 (92)	0.30
≥30	12 (13)	8 (9)		7 (15)	4 (8)	
Unknown	1	0		2	1	
Lymph node status						
N0	70 (76)	76 (87)	0.05	45 (90)	43 (84)	0.39
N^+	22 (24)	11 (13)		5 (10)	8 (16)	
HER2						
Negative	85 (97)	84 (99)	0.33	38 (79)	42 (89)	0.17
Positive	3 (3)	1 (1)		10 (21)	5 (11)	
Unknown	4	2		2	4	
NHG						
I	21 (26)	25 (30)	0.55	6 (12)	16 (36)	0.02
II	47 (59)	50 (60)		33 (67)	24 (55)	
III	12 (15)	8 (10)		10 (20)	4 (9)	
Unknown	12	4		1	7	

ER, estrogen receptor; NHG, Nottingham histological grade; PR, progesterone receptor; RAB6C, Ras-related protein Rab-6C.

Table V. Distant recurrence rate in the independent cohort for high RAB6C compared with low RAB6C stratified by hormone receptor status.

	Number of patients/events		Univariable HR (95% CI)		Multivariable[a] HR (95% CI)	
Hormone receptor status	$RAB6C^+$	$RAB6C^-$	$RAB6C^+$ vs. $RAB6C^-$	P-value	$RAB6C^+$ vs. $RAB6C^-$	P-value
All	204/49	265/73	0.82 (0.57-1.18)	0.29		
ER^+	153/32	157/40	0.77 (0.48-1.22)	0.27		
ER^-	30/13	68/23	1.30 (0.66-2.56)	0.45		
ER^+/PR^+	87/23	92/20	1.20 (0.66-2.19)	0.55	1.31 (0.69-2.48)	0.41
ER^+/PR^-	51/5	50/17	0.24 (0.09 0.66)	<0.01	0.17 (0.05-0.60)	<0.01

[a]Multivariable analysis confined to ER^+ tumors, including age, tumor size, lymph node status, HER2, grade, PR and the interaction term PR x RAB6C. Low RAB6C was the referent (with HR=1) in each subgroup analysis. ER, estrogen receptor; HR, hazard ratio; PR, progesterone receptor; RAB6C, Ras-related protein Rab-6C.

patients with ER^+/PR^+ tumors (HR, 1.31; 95% CI, 0.69-2.48; P=0.41; Table V).

Discussion

To the best of our knowledge, the current study is the first to assess *RAB6C* in a dataset of clinical breast cancer tissue. The prognostic significance was identified using a dataset containing gene copy numbers and further investigations were performed at the protein level in a second independent cohort. The results indicated that high RAB6C expressions decreased the distant recurrence risk of patients, a finding that was most significant in the ER^+/PR^- subgroup. Many of these patients receive both endocrine therapy and chemotherapy, but certain

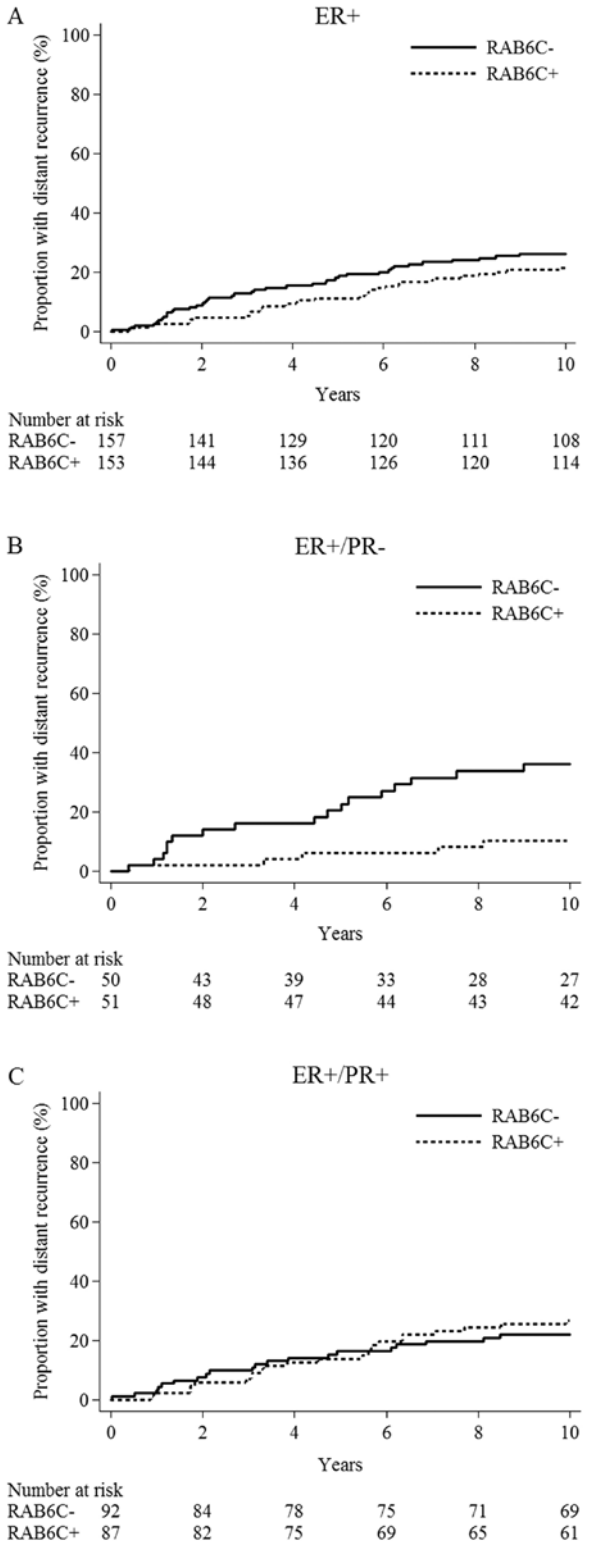

Figure 3. Cumulative distant recurrence risk in relation to the protein expression of RAB6C in patients of the independent cohort. (A) ER^+. HR, 0.77 (95% CI, 0.48-1.22; P=0.27). (B) ER^+/PR^-. HR, 0.24 (95% CI, 0.09-0.66; P=0.005). (C) ER^+/PR^+. HR, 1.20 (95% CI, 0.66-2.19; P=0.55). ER, estrogen receptor; HR, hazard ratio; PR, progesterone receptor; RAB6C, Ras-related protein Rab-6C.

individuals may exhibit a good prognosis even when receiving reduced systemic treatment. In the cohorts of the present study, all patients were systemically untreated, which enabled for the analysis of the natural course associated with RAB6C. The results of the present study may contribute to the understanding of biological heterogeneity within ER^+/PR^- tumors and the identification of potentially overtreated patients.

Variations in gene copy number were analyzed in a dataset of 199 ER^+ tumors, after which the 20 most varied SNPs were selected and tested for prognostic significance. To counteract the problem of multiple testing, the P-value for each SNP was assessed at a significance level of $\alpha/20=0.0025$ according to the principle of Bonferroni's correction. Furthermore, after applying the Cox regression model, *RAB6C* was identified as a prognostic marker.

The identification of RAB6C as a tumor marker with prognostic significance in the current study was supported by previous literature. Bhat *et al* (22,23) used next generation sequencing and data from independent published studies to identify *RAB6C* as one of nine genes with promoter methylation. The high sensitivity and specificity of this could discriminate between normal, premalignant or tumor tissues in cervical tissues and in head and neck squamous cell carcinoma. Furthermore, hypermethylation leading to the downregulation of *RAB6C* was associated with a poorer survival (22,23).

The function of *RAB6C* is yet to be fully elucidated. However, the gene is known to be a member of the RAB6 family, which is highly conserved. By gene duplication and splicing during evolution, RAB6 has diverged to three different isoforms; *RAB6A, RAB6B* and *RAB6C*. *RAB6A* has been subjected to splicing, generating two further variants, *RAB6A* and *RAB6A*'. *RAB6C* was most likely generated by the retrotransposition of mRNA from *RAB6A*' and therefore consists of a single exon (24). This retrotransposition theory is also supported by the fact that *RAB6C* and *RAB6A*' genes exhibit an identity of 97%. However, the proteins that the genes encode seem to have different functions. As far as we know, ER and PR are not target genes of RAB6C, but RAB6C has been demonstrated to be directly targeted by p53 and several experimental studies have indicated that high RAB6C expressions may be a favorable prognostic and predictive factor for cancer (11,13,19). The expression also seems to be associated with the type of tissue. Cells with a high metastatic ability possess lower RAB6C levels than MCF-7 cells, which in turn have lower RAB6C expressions than normal tissue (13). Furthermore, RAB6C inhibits cell proliferation, invasion and metastases (13), and promotes apoptosis (11). Shan *et al* (19) revealed that RAB6C may be involved in chemotherapeutic resistance. Cancer cells expressing RAB6C have also demonstrated an increased sensitivity to several anti-cancer drugs, including doxorubicin, paclitaxel, vinblastine and vincristine (19). Unlike other proteins of the RAB6 family, which are located in the Golgi apparatus serving to regulate protein transport, RAB6C is primarily localized to the centrosome and is involved in cell division (24). This distinguished function of RAB6C may contribute to the prognostic impact that was identified in the current study.

We identified prognostic significance of *RAB6C* based on gene copy number with data from a public data set. Since gene copy number variation is an indicator of genomic instability, which per se implies poor prognosis, it is important to consider also the gene expression. Therefore, we also analyzed RAB6C protein expression in an independent cohort. We did not have data on both gene copy number and gene expression in either cohort, which impeded a correlation analysis between gene copy number

and gene expression. Gene expression analysis of RAB6C is complicated due to the fact that it consists of a single exon and several gene expression microarrays are not able to distinguish between mRNA expression of RAB6A and RAB6C (24,25). In order to distinguish between RAB6A and RAB6C, protein expression analysis seems to be a more reliable method.

RAB6C is located within a genomic region with large-scale copy number polymorphism, contributing to genomic variation between normal humans (26). The RAB6C-like protein is expressed by a gene located in the same chromosomal region within a distance of 1.38 Mb from *RAB6C* (24). The antibody that was used in the current study has been confirmed to detect RAB6C. However, the proteins are 100% identical according to NCBI's protein database, which uses the BLAST tool (27) and differ at three amino acids according to the database of Ensembl (28). Thus, RAB6C and RAB6C-like protein are very similar and the gene products may possess the same function.

In the public dataset and the independent cohort used in the current study, an association was determined between grade and RAB6C, particularly among ER^+/PR^- tumors. In the independent cohort, the current study also analyzed the association between RAB6C and the three components of grading (pleomorphism, tubule formation and mitosis). The results revealed a negative correlation between RAB6C and each of the components, including mitosis ($P<0.001$). This seems reasonable, since RAB6C has been demonstrated to serve a role in DNA replication and it is known that the depletion of RAB6C generates tetraploid cells with supernumerary centrosomes (24).

The present study analyzed the prognostic course associated with RAB6C. Since most ER^+ patients receive systemic therapy and experimental studies have indicated that RAB6C may be involved in chemotherapeutic resistance, a treatment predictive value should be investigated in future study.

Acknowledgements

The authors would like to thank data manager Mrs. Ulla Johansson (Regional Cancer Center of Stockholm Gotland, Stockholm, Sweden) for updating the database, technician Mrs. Birgitta Holmlund (Department of Oncology, Linköping University Hospital, Linköping, Sweden) for technical assistance, and Dr Najme Wall (Department of Oncology, Linköping University Hospital, Linköping, Sweden), Dr Hans Olsson (Department of Pathology, Linköping University Hospital, Linköping, Sweden) and Dr Dennis Sgroi (Department of Pathology, Massachusetts General Hospital, Boston, MA, USA) for determining tumor grades.

Funding

The present study was supported by grants obtained from the Swedish Cancer Society (OS; grant no. 17-0479), The Cancer Research Foundations of Radiumhemmet (TF), the Cancer Society in Stockholm (TF) and the King Gustav V Jubilee Clinical Research Foundation (TF; grant no. 181093), the Onkologiska klinikerna i Linköpings forskningsfond (HF; grant no. 2016-06-21), ALF grants Region Östergötland (OS; grant no. LIO-795201) and the County Council of Östergötland (HF; grant no. LIO-625491). Funding sources did not have any role in the study design, in the collection, analysis, interpretation of data, in the writing of the manuscript or in the decision to submit the manuscript for publication.

Availability of data and materials

The public data set with accession number GSE10099 analyzed during the present study is available in the NCBI GEO repository (http://www.ncbi.nlm.nih.gov/). The datasets from the independent cohort used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HF, JC and OS conceived and designed the present study. TF and BN provided the study materials, collected clinical follow-up data and hormone receptor data from the patients, and performed the clinical interpretation. TB, JS and HF performed the experiments and scored RAB6C. HF performed statistical analysis and drafted the manuscript. HF, JC, OS, TB, JS, BN and TF interpreted the results, critically revised the manuscript and approved the final version.

Ethics approval and consent to participate

The present study was performed in accordance with the Declaration of Helsinki. For the independent cohort, an ethical approval for the use of tumor material was provided by the local Ethical Committee of Karolinska University Hospital (approval no. KI 97-451 with amendments 030201 and 171027). According to the approval that was received, patient informed consent was not required.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Perou CM, Sørlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA, *et al*: Molecular portraits of human breast tumours. Nature 406: 747-752, 2000.
2. Gruvberger S, Ringnér M, Chen Y, Panavally S, Saal LH, Borg A, Fernö M, Peterson C and Meltzer PS: Estrogen receptor status in breast cancer is associated with remarkably distinct gene expression patterns. Cancer Res 61: 5979-5984, 2001.
3. Zhang Y, Martens JW, Yu JX, Jiang J, Sieuwerts AM, Smid M, Klijn JG, Wang Y and Foekens JA: Copy number alterations that predict metastatic capability of human breast cancer. Cancer Res 69: 3795-3801, 2009.
4. Grann VR, Troxel AB, Zojwalla NJ, Jacobson JS, Hershman D and Neugut AI: Hormone receptor status and survival in a population-based cohort of patients with breast carcinoma. Cancer 103: 2241-2251, 2005.
5. Early Breast Cancer Trialists' Collaborative Group (EBCTCG), Davies C, Godwin J, Gray R, Clarke M, Cutter D, Darby S, McGale P, Pan HC, Taylor C, *et al*: Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: Patient-level meta-analysis of randomised trials. Lancet 378: 771-784, 2011.

6. Thakkar JP and Mehta DG: A review of an unfavorable subset of breast cancer: Estrogen receptor positive progesterone receptor negative. Oncologist 16: 276-285, 2011.
7. Arpino G, Weiss H, Lee AV, Schiff R, De Placido S, Osborne CK and Elledge RM: Estrogen receptor-positive, progesterone receptor-negative breast cancer: Association with growth factor receptor expression and tamoxifen resistance. J Natl Cancer Inst 97: 1254-1261, 2005.
8. Viale G, Regan MM, Maiorano E, Mastropasqua MG, Dell'Orto P, Rasmussen BB, Raffoul J, Neven P, Orosz Z, Braye S, *et al*: Prognostic and predictive value of centrally reviewed expression of estrogen and progesterone receptors in a randomized trial comparing letrozole and tamoxifen adjuvant therapy for postmenopausal early breast cancer: BIG 1-98. J Clin Oncol 25: 3846-3852, 2007.
9. Cancer Genome Atlas Network: Comprehensive molecular portraits of human breast tumours. Nature 490: 61-70, 2012.
10. Haverty PM, Fridlyand J, Li L, Getz G, Beroukhim R, Lohr S, Wu TD, Cavet G, Zhang Z and Chant J: High-resolution genomic and expression analyses of copy number alterations in breast tumors. Genes Chromosomes Cancer 47: 530-542, 2008.
11. Tian K, Wang Y and Xu H: WTH3 is a direct target of the p53 protein. Br J Cancer 96: 1579-1586, 2007.
12. Tian K, Wang Y, Huang Y, Sun B, Li Y and Xu H: Methylation of WTH3, a possible drug resistant gene, inhibits p53 regulated expression. BMC Cancer 8: 327, 2008.
13. Gan L, Zuo G, Wang T, Min J, Wang Y, Wang Y and Lv G: Expression of WTH3 in breast cancer tissue and the effects on the biological behavior of breast cancer cells. Exp Ther Med 10: 154-158, 2015.
14. Wang Y, Klijn JG, Zhang Y, Sieuwerts AM, Look MP, Yang F, Talantov D, Timmermans M, Meijer-van Gelder ME, Yu J, *et al*: Gene-expression profiles to predict distant metastasis of lymph-node-negative primary breast cancer. Lancet 365: 671-679, 2005.
15. Rutqvist LE and Johansson H; Stockholm Breast Cancer Study Group: Long-term follow-up of the randomized Stockholm trial on adjuvant tamoxifen among postmenopausal patients with early stage breast cancer. Acta Oncol 46: 133-145, 2007.
16. Rutqvist LE and Johansson H: Long-term follow-up of the Stockholm randomized trials of postoperative radiation therapy versus adjuvant chemotherapy among 'high risk' pre- and post-menopausal breast cancer patients. Acta Oncol 45: 517-527, 2006.
17. Jerevall PL, Jansson A, Fornander T, Skoog L, Nordenskjöld B and Stål O: Predictive relevance of HOXB13 protein expression for tamoxifen benefit in breast cancer. Breast Cancer Res 12: R53, 2010.
18. Wrange O, Nordenskjöld B and Gustafsson JA: Cytosol estradiol receptor in human mammary carcinoma: An assay based on isoelectric focusing in polyacrylamide gel. Anal Biochem 85: 461-475, 1978.
19. Shan J, Mason JM, Yuan L, Barcia M, Porti D, Calabro A, Budman D, Vinciguerra V and Xu H: Rab6c, a new member of the rab gene family, is involved in drug resistance in MCF7/AdrR cells. Gene 257: 67-75, 2000.
20. Shan J, Yuan L, Budman DR and Xu HP: WTH3, a new member of the Rab6 gene family, and multidrug resistance. Biochim Biophys Acta 1589: 112-123, 2002.
21. Uhlén M, Björling E, Agaton C, Szigyarto CA, Amini B, Andersen E, Andersson AC, Angelidou P, Asplund A, Asplund C, *et al*: A human protein atlas for normal and cancer tissues based on antibody proteomics. Mol Cell Proteomics 4: 1920-1932, 2005.
22. Bhat S, Kabekkodu SP, Jayaprakash C, Radhakrishnan R, Ray S and Satyamoorthy K: Gene promoter-associated CpG island hypermethylation in squamous cell carcinoma of the tongue. Virchows Arch 470: 445-454, 2017.
23. Bhat S, Kabekkodu SP, Varghese VK, Chakrabarty S, Mallya SP, Rotti H, Pandey D, Kushtagi P and Satyamoorthy K: Aberrant gene-specific DNA methylation signature analysis in cervical cancer. Tumour Biol 39: 1010428317694573, 2017.
24. Young J, Menetrey J and Goud B: RAB6C is a retrogene that encodes a centrosomal protein involved in cell cycle progression. J Mol Biol 397: 69-88, 2010.
25. Williams TK, Yeo CJ and Brody J: Does this band make sense? Limits to expression based cancer studies. Cancer Lett 271: 81-84, 2008.
26. Sebat J, Lakshmi B, Troge J, Alexander J, Young J, Lundin P, Månér S, Massa H, Walker M, Chi M, *et al*: Large-scale copy number polymorphism in the human genome. Science 305: 525-528, 2004.
27. Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W and Lipman DJ: Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acids Res 25: 3389-3402, 1997.
28. Yates A, Akanni W, Amode MR, Barrell D, Billis K, Carvalho-Silva D, Cummins C, Clapham P, Fitzgerald S, Gil L, *et al*: Ensembl 2016. Nucleic Acids Res 44: D710-D716, 2016.