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Polymorphisms in predicted microRNA-binding sites in integrin genes and breast cancer: ITGB4 as prognostic marker

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Integrins control the cell attachment to the extracellular matrix and play an important role in mediating cell proliferation, migration and survival. A number of important cancer-associated integrin genes can be regulated by microRNAs (miRNAs) that bind to their target sites in the 3′ untranslated regions. We examined the effect of single-nucleotide polymorphisms (SNPs) in predicted miRNA target sites of six integrin genes (*ITGA3*, *ITGA6*, *ITGAv*, *ITGB3*, *ITGB4* and *ITGB5*) on breast cancer (BC) risk and clinical outcome. Six SNPs were genotyped in 749 Swedish incident BC cases with detailed clinical data and up to 15 years of follow-up together with 1493 matched controls. We evaluated associations between genotypes and BC risk and clinical tumour characteristics. Survival probabilities were compared between different subgroups. As a novel finding, several SNPs seemed to associate with the hormone receptor status. The strongest association was observed between the A allele of the SNP rs743554 in the *ITGB4* gene and oestrogen receptor-negative tumours [odds ratio 2.09, 95% confidence intervals (CIs) 1.19–3.67]. The same SNP was associated with survival. The A allele carriers had a worse survival compared with the wild-type genotype carriers (hazard ratio 2.11, 95% CIs 1.21–3.68). The poor survival was significantly associated with the aggressive tumour characteristics: high grade, lymph node metastasis and high stage. None of the SNPs was significantly associated with BC risk. As the *ITGB4* SNP seems to influence tumour aggressiveness and survival, it may have prognostic value in the clinic.

Introduction

It is well established that genetic variation in addition to the mutations in the known breast cancer (BC) susceptibility genes BRCA1 and BRCA2 affect individual's risk of BC (1). Recently, it has become increasingly clear that survival in BC has an inherited component (2–5). While cancer risk is related to defects in cell cycle control and DNA integrity (6), survival is dependent on tumour progression and metastasis (5,7). The critical early stages of metastasis include increased cell motility and production of matrix-degrading proteases (7).

Integrins comprise a large family of cell surface receptors. They control cell attachment to the extracellular matrix (ECM) and play a key role in mammary gland biology, where integrins are present in all cell types within the gland (8,9). In addition to their adhesive functions, integrins can activate intracellular signalling pathways that control growth, differentiation, apoptosis, cell motility, migration and survival (8–10).

Integrins are heterodimers composed of two non-covalently linked type 1 transmembrane subunits α and β. In mammals, 18 α and 8 β subunits associate in various combinations to form at least 24 integrins that bind specific ECM components (9–11). Binding of the ECM ligands to integrins is followed by the recruitment of several signalling and adaptor proteins to the integrin cytoplasmic domain and the activation of the downstream signalling pathways.

Tumour cells use integrins for proliferation, migration and survival depending on their cellular context and their stage of progression (8,9,12). Whereas neoplastic cells enhance the expression of integrins that favour their proliferation, survival and migration, they tend to diminish the expression of integrins that exert the opposite effect (12). *De novo* expression or up-regulation of certain integrins including αvβ3, α6β1 and α6β4 is associated with a metastatic phenotype and increased motility in a variety of cancer cells, such as melanoma, breast and prostate carcinoma. Reduced expression of other integrins, such as α2β1, in primary BC cells has been correlated with increased invasion and metastasis (8,12).

The 3′ untranslated regions (UTRs) are involved in the regulation of gene expression at multiple levels: at the pre-messenger RNA (mRNA) level, 3′ UTRs are involved in the mRNA 3′ end formation and polyadenylation, whereas at the mature mRNA level, they determine properties such as mRNA stability/degradation, nuclear export, subcellular localization and translation efficiency (13). MicroRNAs (miRNAs), which bind to imperfect complementary sites within the 3′ UTRs of their mRNA targets, have been proposed to contribute to tumourigenesis because they can either function as tumour suppressors or oncogenes (14). A number of important cancer-associated integrins can be regulated by miRNAs and therefore may contribute to altered expression of integrin genes (15).

Genetic variation in the regulatory 3′ UTR of the integrin genes may affect gene expression and thus BC susceptibility as well as tumour aggressiveness and survival of the BC patients. In the present case–control study, we investigated the effect of six single-nucleotide polymorphisms (SNPs) in the 3′ UTRs of six integrin genes on BC risk, clinical tumour characteristics and patient survival. We used a large Swedish study population with detailed clinical data and a follow-up time of up to 15 years.

Materials and methods

Study population

The analyses were performed on genomic DNA from 749 Swedish BC cases together with 1493 controls. The cases with the age- and gender-matched controls were drawn from the population-based Västerbotten intervention project and the mammary screening project, which contain blood samples collected between January 1990 and January 2001 from an ethnically homogeneous population living in a geographically defined region in North Sweden (16). Prospective cases were identified from the cohorts by record linkage to the regional cancer registry. The controls were selected from the same cohort as the corresponding case. They were matched with the case by age at baseline (±6 months) and the time of sampling (±2 months). The controls had to be alive at the time of diagnosis of the corresponding case and without any previous cancer diagnosis, except carcinoma *in situ* of cervix uteri. All participants gave informed consent to the use of their samples for research purpose. The blood samples were stored at −80°C until the time of sample selection and DNA isolation for genotyping analyses. The samples were randomly divided on the 96-well plates and the genotyping was performed blinded by the case–control status of each sample. Clinical data for the unselected BC cases were retrieved from the registry managed by the Northern Sweden Breast Cancer Group (Table I). Follow-up was performed until 26 April 2007. Information about the date of death was collected from the Swedish population

Abbreviations: BC, breast cancer; CI, confidence interval; ECM, extracellular matrix; ER, oestrogen receptor; HR, hazard ratio; miRNA, microRNA; mRNA, messenger RNA; OR, odds ratio; SNP, single-nucleotide polymorphism; UTR, untranslated region; WT, wild-type.

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Table I. Characteristics of the Swedish BC samples at diagnosis

Characteristics	BC patients, *n* (%)
Age at diagnosis, mean (range, SD)	58.1 (30.9–76.1, 8.67)
ER	
Positive	200 (26.7)
Negative	80 (10.7)
Missing data	469 (62.6)
PR	
Positive	177 (23.6)
Negative	97 (13.0)
Missing data	475 (63.4)
Hormone receptor combination	
ER+/PR+	164 (21.9)
ER+/PR−	31 (4.1)
ER−/PR+	13 (1.7)
ER−/PR−	66 (8.8)
Missing data	475 (63.4)
Tumor size in cm	
≤2 cm	486 (64.9)
>2 cm	214 (28.6)
Missing data	49 (6.5)
Histologic grade	
1	155 (20.7)
2	342 (45.7)
3	215 (28.7)
Missing data	37 (4.9)
Regional lymph node metastasis	
Negative	450 (60.1)
Positive	209 (27.9)
Missing data	90 (12.0)
Stage at diagnosis	
0	2 (0.3)
I	392 (52.3)
II	310 (41.4)
III	25 (3.3)
IV	14 (1.9)
Missing data	6 (0.8)
Distant metastasis	
Negative	728 (97.2)
Positive	13 (1.7)
Missing data	8 (1.1)

register with a BC-specific follow-up until 31 December 2004. The median follow-up time for BC-specific survival was 4.7 years. The study was approved by the ethical committee of Karolinska Institute Syd and Umeå University.

SNP screening by sequencing

We screened polymorphisms in the 3′ UTRs of 10 integrin genes: *ITGA3*, *ITGA5*, *ITGA6*, *ITGAv*, *ITGB1*, *ITGB3*, *ITGB4* and *ITGB5*, *ITGB6* and *ITGB8*. Putative miRNA-binding sites were determined using the online available tools such as microInspector (http://mirna.imbb.forth.gr/microinspector/), PicTar (http://pictar.bio.nyu.edu/) and TargetScan (http://www.targetscan.org/).

To confirm the presence and frequency of the SNPs and to obtain standards for genotyping with TaqMan, a randomly chosen set of 32 BC samples was investigated by sequencing. The polymerase chain reaction and the sequencing reaction were performed as described earlier by Vaclavicek *et al.* (17). Primer sequences and annealing temperatures are available from the corresponding author on request.

TaqMan allelic discrimination

The TaqMan allelic discrimination method was used to genotype the six selected SNPs. TaqMan assays (Assay-on-demand) were ordered from Applied Biosystems. The reaction was performed in 5 µl using 225 nM each primer, 50 nM each probe and 2.5 µl TaqMan Universal 2× PCR Master Mix (Applied Biosystems, Foster City, CA). Polymerase chain reaction conditions were as described previously (17). The samples were read and analysed in an ABI Prism 7900HT Sequence Detection System using SDS 1.2 software (Applied Biosystems). The large sample set for genotyping contained 104 duplicate samples (four duplicates on each 96-well plate), which represent ∼5% of all samples. The results of the duplicate samples agreed with each other.

Statistical analysis

The observed genotype frequencies in the controls were tested for Hardy–Weinberg equilibrium and the difference between the observed and expected frequencies was tested for significance using the χ^2 test. Statistical significance for the differences in the genotype frequencies between the BC cases and controls was determined by the Wald χ^2 test of heterogeneity with two degrees of freedom. Odds ratios (ORs) and 95% confidence intervals (CIs) for association between genotypes and BC risk and tumour characteristics were calculated by logistic regression (PROC LOGISTIC, SAS Version 9.1; SAS Institute, Cary, NC). For a polymorphism with a variant allele frequency between 15 and 45%, the study had >90% power to detect an OR of 1.40 at a significance level of 0.05 (PS-software for power and sample size calculation, http://biostat.mc.vanderbilt.edu/twiki/bin/view/Main/PowerSampleSize). The survival curves for BC-specific survival were derived by the Kaplan–Meier method (PROC LIFETEST, SAS Version 9.1; SAS Insitute). The overall relative risk of death by BC according to the genotype was estimated as hazard ratio (HR) using Cox regression (PROC PHREG, SAS Version 9.1; SAS Institute). Censoring events were death by another cause than BC, moving out of the study and 31 December 2004. The HRs were also calculated within subgroups of cases with a similar manifestation of a clinical factor. Furthermore, the HR for the overall risk was adjusted for the clinical factors [oestrogen receptor (ER) status, progesterone receptor (PR) status, tumour size, lymph node metastasis and histologic grade] to determine the value of the genotypes as an independent prognostic marker.

Results

Selection of SNPs

A randomly chosen set of 32 Swedish BC samples was used to screen the 3′ UTRs for SNPs and to select positive controls for subsequent TaqMan allelic discrimination assays. In 7 of 10 genes, we detected 12 SNPs in the 3′ UTRs with a frequency ≥10% (*ITGA3*: one SNP, *ITGA6*: one SNP, *ITGAv*: three SNPs, *ITGB1*: one SNP, *ITGB3*: one SNP, *ITGB4*: two SNPs and *ITGB5*: two SNPs) (supplementary Table I, available at *Carcinogenesis* Online). When we detected more than one SNP in a gene and these SNPs were in high linkage disequilibrium, we only selected one SNP for further investigation (supplementary Table II, available at *Carcinogenesis* Online). When we predicted possible targets of human miRNAs in the integrin genes using the online databases microInspector, PicTar and TargetScan, many possible binding sites in the 3′ UTRs of integrins were revealed. We did not only select SNPs located within the 'miRNA seed' which encompasses the first two to eight nucleotides of the target sequence but also SNPs that were located in the whole mature miRNA sequence. The following SNPs within putative binding sites of miRNAs were selected for genotyping: rs1062484 *(ITGA3)*, rs17664 *(ITGA6)*, rs11902171 *(ITGAv)*, rs17468 *(ITGB1)*, rs3809865 *(ITGB3)*, rs743554 *(ITGB4)* and rs2675 *(ITGB5)*. The TaqMan assay for the SNP rs17468 in the *ITGB1* gene did not work properly and therefore the SNP was excluded from our study. For the *ITGB4* SNP rs9367, the TaqMan assay could not be designed and we analysed rs743554 instead. The wild-type (WT) alleles of all investigated SNPs were located within putative miRNA-binding sites. Although there seemed to be no differences in miRNA–mRNA binding characteristics in the form of free energy between the WT and the variant allele, for all SNPs the possible number of miRNA targets varied between the WT and the variant allele (rs1062484: WT 11, variant 17; rs17664: WT 3, variant 4; rs11902171: WT 3, variant 0; rs3809865: WT 1, variant 4; rs743554: WT 1, variant 0 and rs2675: WT 4, variant 5) (supplementary Table III, available at *Carcinogenesis* Online). Only the SNPs rs11902171 (*ITGAv*) and rs3809865 (*ITGB3*) were located within the miRNA seed (mir-30e-3p, mir-382, mir30a-3p and mir-26b, mir-330, let-7b, mir-324-5b and mir-524, respectively).

No association of SNPs with BC susceptibility

No differences in the allele or genotype frequencies between the BC cases and controls were detected (supplementary Table IV, available at *Carcinogenesis* Online). For the SNP rs17664 (*ITGA6*), a significant global *P* value ($P = 0.02$) was observed; however, for an unknown reason, the genotype distribution in the controls deviated

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Table II. Associations of the integrin genotypes of the cases with hormone receptor status in the Swedish population

Tumour characteristics											
Gene	Genotype	ER			PR			ER/PR combination			
		Positive	Negative	OR (95% CI)	Positive	Negative	OR (95% CI)	ER+/PR+	ER+/PR− or ER−/PR+	OR (95% CI)	ER−/PR−	OR (95% CI)
ITGA3 rs1062484	CC	101 (51.8)	35 (45.5)	1.00	85 (49.7)	48 (50.5)	1.00	80 (50.3)	23 (53.5)	1.00	30 (46.9)	1.00
	CT	82 (42.1)	35 (45.5)	1.23 (0.71–2.14)	77 (45.0)	38 (40.0)	0.87 (0.52–1.48)	70 (44.0)	17 (39.5)	0.85 (0.42–1.71)	28 (43.8)	1.07 (0.58–1.96)
	TT	12 (6.1)	7 (9.1)	1.68 (0.61–4.61)	9 (5.3)	9 (9.5)	1.77 (0.66–4.76)	9 (5.7)	3 (7.0)	1.16 (0.29–4.64)	6 (9.4)	1.78 (0.58–5.42)
ITGA6 rs17664	AA	67 (34.2)	23 (29.1)	1.00	57 (32.8)	31 (32.3)	1.00	53 (32.9)	16 (36.4)	1.00	19 (29.2)	1.00
	AG	104 (53.1)	47 (59.9)	1.32 (0.73–2.37)	95 (54.6)	53 (55.2)	1.03 (0.59–1.78)	88 (54.7)	21 (47.7)	0.79 (0.38–1.65)	39 (60.0)	1.24 (0.65–2.36)
	GG	25 (12.8)	9 (11.4)	1.05 (0.43–2.57)	22 (12.6)	12 (12.5)	1.00 (0.44–2.30)	20 (12.4)	7 (15.9)	1.16 (0.42–3.24)	7 (10.8)	0.98 (0.36–2.67)
ITGAv rs11902171	GG	99 (50.5)	48 (60.0)	1.00	90 (51.7)	54 (55.7)	1.00	82 (50.9)	23 (52.3)	1.00	39 (59.1)	1.00
	GC	80 (40.8)	31 (38.8)	0.80 (0.47–1.37)	70 (40.2)	39 (40.2)	0.93 (0.55–1.55)	65 (40.4)	18 (40.9)	0.99 (0.49–1.98)	26 (39.4)	0.84 (0.47–1.52)
	CC	17 (8.7)	1 (1.3)	**0.12 (0.02–0.94)**	14 (8.1)	4 (4.1)	0.48 (0.15–1.52)	14 (8.7)	3 (6.8)	0.76 (0.20–2.89)	1 (1.5)	0.15 (0.02–1.18)
	GC + CC	97 (32.7)	32 (24.8)	0.68 (0.40–1.15)	84 (48.3)	43 (44.3)	0.85 (0.52–1.41)	79 (49.1)	21 (47.7)	0.95 (0.49–1.85)	27 (40.9)	0.72 (0.40–1.28)
ITGB3 r3809865	AA	84 (42.2)	37 (46.3)	1.00	76 (43.2)	42 (43.4)	1.00	71 (43.6)	16 (36.4)	1.00	31 (47.0)	1.00
	AT	88 (44.2)	33 (41.3)	0.85 (0.49–1.49)	77 (43.8)	42 (43.4)	0.99 (0.58–1.68)	71 (43.6)	21 (47.7)	1.31 (0.63–2.72)	27 (40.9)	0.87 (0.47–1.61)
	TT	27 (13.6)	19 (12.5)	0.84 (0.37–1.91)	23 (13.1)	13 (13.4)	1.02 (0.47–2.23)	21 (12.9)	7 (15.9)	1.48 (0.54–4.07)	8 (12.1)	0.87 (0.35–2.18)
ITGB4 rs743554	GG	150 (77.3)	49 (62.0)	1.00	129 (74.1)	66 (70.2)	1.00	122 (75.8)	32 (76.2)	1.00	41 (63.1)	1.00
	GA	42 (21.7)	25 (31.7)	**1.82 (1.01–3.29)**	42 (24.1)	24 (25.5)	1.18 (0.62–2.00)	37 (23.0)	9 (21.4)	0.93 (0.41–2.12)	20 (63.1)	1.61 (0.84–3.08)
	AA	2 (1.0)	5 (6.3)	**7.65 (1.44–40.7)**	3 (1.7)	4 (4.3)	2.60 (0.57–11.98)	2 (1.2)	1 (2.4)	1.91 (0.17–21.70)	4 (6.2)	**5.95 (1.05–33.70)**
	GA + AA	44 (22.7)	30 (38.0)	**2.09 (1.19–3.67)**	45 (25.9)	28 (29.8)	1.22 (0.70–2.12)	39 (24.2)	10 (23.8)	0.98 (0.44–2.17)	24 (36.9)	1.83 (0.99–3.40)
ITGB5 rs2675	AA	144 (73.5)	49 (62.0)	1.00	128 (73.6)	61 (63.5)	1.00	121 (75.2)	27 (61.4)	1.00	41 (63.1)	1.00
	AC	47 (24.0)	28 (35.5)	1.75 (0.99–3.09)	40 (23.0)	34 (35.4)	**1.78 (1.03–3.09)**	35 (21.7)	16 (36.4)	2.05 (0.99–4.23)	23 (35.5)	**1.94 (1.03–3.66)**
	CC	5 (2.6)	2 (2.5)	1.18 (0.22–6.25)	6 (3.4)	1 (1.1)	0.35 (0.04–2.97)	5 (3.1)	1 (2.2)	0.90 (0.10–7.99)	1 (1.5)	0.59 (0.07–5.20)
	AC + CC	52 (22.7)	30 (38.0)	1.70 (0.97–2.95)	46 (25.9)	35 (29.8)	1.60 (0.94–2.72)	40 (24.8)	17 (38.6)	1.91 (0.94–3.85)	24 (36.9)	1.77 (0.96–3.28)

Because of missing clinical data, the number of cases in Table II were less than in supplementary Table IV (available at *Carcinogenesis* Online). The ORs were considered statistically significant when the 95% CIs did not overlap unity.

slightly from the Hardy–Weinberg equilibrium ($P = 0.03$). All other genotype distributions among the controls followed Hardy–Weinberg equilibrium and all observed allele frequencies were approximately concordant with the ones published for Caucasians at the NCBI and HapMap databases.

Since integrins are heterodimers consisting of one α and one β subunit, we wanted to study interactions of the SNPs in the genes coding for the subunits of α6β4 (rs17664/rs743554), αvβ3 (rs11902171/rs3809865) and αvβ5 (rs11902171/rs2675). No significant effect on BC risk was observed for any gene combination (global P value = 0.27, 0.52 and 0.22, respectively; data not shown).

Association of SNPs with breast tumour characteristics at the time of diagnosis

When we stratified the BC cases by all tumour characteristics listed in Table I, some associations in relation to the hormone receptor status emerged (Table II). For the SNP rs743554 in the *ITGB4* gene, the carriers of the GA and AA genotypes had more often ER− tumours than carriers of the WT GG genotype (OR 2.09, 95% CI 1.19–3.67). The carriers of the AC genotype of the SNP rs2675 (*ITGB5*) had more often hormone receptor-negative tumours than the carriers of the WT AA genotype. However, no effect of the rare CC genotype on hormone receptor status was observed and the association between the C allele carrier status and hormone receptor status was of borderline significance. The carriers of the rare CC genotype of the SNP rs11902171 (*ITGAv*) were more likely to have ER+ tumours (OR 0.12, 95% CI 0.02–0.94) than carriers of the WT genotype GG. No associations between the genotypes and the tumour characteristics such as tumour size, histologic grade, regional lymph node metastasis and stage were observed (supplementary Table V, available at *Carcinogenesis* Online).

Association with BC-specific survival

The survival analysis stratified by the genotypes of the studied SNPs in all cases showed that the carriers of the GA and AA genotypes of the SNP rs743554 in the *ITGB4* gene had worse BC-specific survival compared with the WT genotype GG carriers (HR 2.11, 95% CI 1.21–3.68) (Table III, Figure 1A). The other investigated SNPs were

Table III. BC-specific survival of the selected integrin SNPs in all cases

Survival analysis				
Gene	Genotype	BC-specific survival		
		n_{all}	n_{died} (%)	HR (95% CI)
ITGA3 rs1062484	CC	370	26 (7.0)	1.00
	CT	280	19 (6.8)	0.91 (0.51–1.65)
	TT	55	3 (5.5)	0.79 (0.24–2.61)
ITGA6 rs17664	AA	250	16 (6.4)	1.00
	AG	361	25 (6.9)	1.02 (0.54–1.90)
	GG	101	8 (7.9)	1.17 (0.50–2.73)
ITGAv rs11902171	GG	377	26 (6.9)	1.00
	GC	283	20 (7.1)	0.94 (0.53–1.69)
	CC	54	3 (5.6)	0.69 (0.21–2.27)
	GC + CC	337	23 (6.9)	0.90 (0.51–1.58)
ITGB3 rs3809865	AA	308	24 (7.8)	1.00
	AT	326	19 (5.8)	0.78 (0.43–1.42)
	TT	80	6 (7.5)	0.91 (0.37–2.22)
ITGB4 rs743554	GG	506	29 (5.7)	1.00
	GA	177	18 (10.2)	**1.95 (1.08–3.51)**
	AA	20	4 (20.0)	**3.38 (1.19–9.61)**
	GA + AA	197	22 (11.2)	**2.11 (1.21–3.68)**
ITGB5 rs2675	AA	522	37 (7.1)	1.00
	AC	171	10 (5.8)	0.76 (0.38–1.54)
	CC	17	2 (11.8)	1.90 (0.46–7.88)
	AC + CC	188	12 (6.4)	0.85 (0.44–1.63)

The HRs were considered statistically significant when the 95% CIs did not overlap unity.

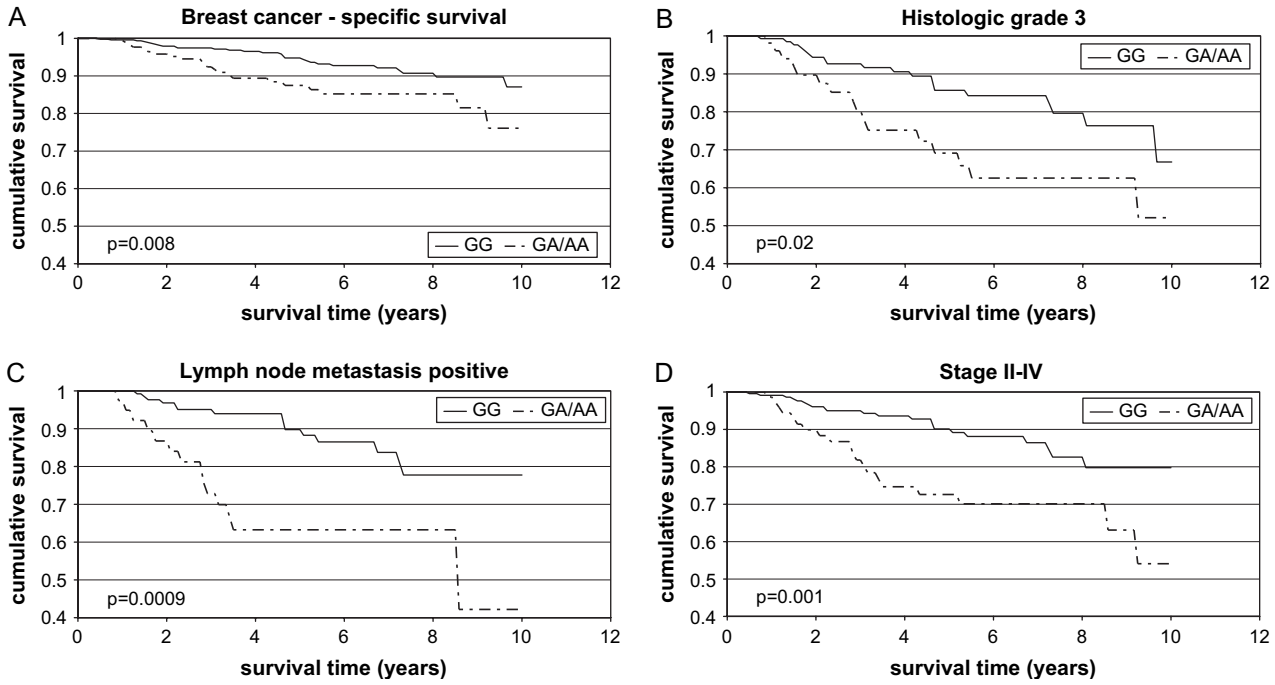

Fig. 1. Survival of the breast cancer patients carrying the GG or the GA/AA genotypes of the SNP rs743554 (ITGB4) after the diagnosis of cancer. (**A**) breast cancer-specific survival, (**B–D**) survival in relation to the following tumour characteristics: histologic grade 3, lymph node metastasis positive, stage II–IV.

Table IV. Survival analysis for the SNP rs743554 in relation to all tumour characteristics at the time of diagnosis

Survival analysis for rs743554				
Survival	Genotype	n_{all}	n_{died} (%)	HR (95% CI)
BC-specific survival	GG	506	29 (5.7)	1.00
	GA + AA	197	22 (11.2)	**2.11 (1.21–3.68)**
PR+/ER+	GG	122	8 (6.6)	1.00
	GA + AA	39	5 (12.8)	2.07 (0.68–6.33)
ER+/PR− or ER−/PR+	GG	32	5 (15.6)	1.00
	GA + AA	10	1 (10.0)	0.67 (0.08–5.78)
PR−/ER−	GG	41	5 (12.2)	1.00
	GA + AA	24	7 (29.2)	2.60 (0.82–8.28)
Tumour size ≤2 cm	GG	332	10 (3.0)	1.00
	GA + AA	132	10 (7.6)	**2.93 (1.22–7.04)**
Tumour size >2 cm	GG	148	16 (10.8)	1.00
	GA + AA	48	10 (20.8)	1.92 (0.87–4.24)
Histologic grades 1 + 2	GG	340	8 (2.4)	1.00
	GA + AA	127	6 (4.7)	2.07 (0.72–5.98)
Histologic grade 3	GG	141	20 (14.2)	1.00
	GA + AA	60	16 (26.7)	**2.26 (1.17–4.37)**
Lymph node metastasis negative	GG	298	11 (3.7)	1.00
	GA + AA	124	6 (4.8)	1.41 (0.52–3.82)
Lymph node metastasis positive	GG	150	15 (10.0)	1.00
	GA + AA	48	14 (29.2)	**3.44 (1.66–7.13)**
Stages 0 + I	GG	267	7 (2.6)	1.00
	GA + AA	109	2 (1.8)	0.82 (0.17–3.96)
Stages II–IV	GG	237	22 (9.3)	1.00
	GA + AA	86	20 (23.3)	**2.69 (1.47–4.93)**

The HRs were considered statistically significant when the 95% CIs did not overlap unity.

not associated with survival (Table III). For the SNP rs743554 (*ITGB4*), we further examined the BC-specific survival in relation to tumour characteristics (Table IV, Figure 1B–D). The survival was worst in women with the GA and AA genotypes, when they had more aggressive tumours (hormone receptor negative, large tumours, high grade, lymph node metastasis positive and high-stage tumours), although in the case of hormone receptor status and tumour size, the effect was not statistically significant. In the case of tumour size, the A allele carrier status seemed to correlate stronger with worse survival in women with smaller tumours than in women with larger tumours. In the multivariate analysis, taking into account all clinical markers of the tumours, the A allele carrier status was significantly associated with survival (HR 2.88, 95% CI 1.36–6.07), indicating the value of the *ITGB4* SNP rs743554 as an independent prognostic marker.

Discussion

Functional polymorphisms, which have an effect on the regulation of gene expression, can contribute to the differences between individuals in susceptibility to and severity of a disease. The effect may be seen by a polymorphism alone or in combination with other polymorphisms. Integrins can be regulated by miRNAs that bind to their target sites in the 3′ UTRs and therefore may contribute to altered expression of the integrin genes (15). Although it is difficult to determine biologically relevant miRNA targets, there is increasing evidence that alterations at the miRNA target sequences contribute to disease, including cancer (14,18,19). Recently, two SNPs located within the recognition sites of several miRNAs in the 3′ UTR of the KIT oncogene have been shown to affect the miRNA–mRNA interactions in papillary thyroid cancer (19).

As a main observation of our study, the A allele of the *ITGB4* SNP rs743554 was associated with the negative hormone receptor status and bad BC-specific survival, especially in women with more aggressive tumours. The multivariate analysis suggested that the *ITGB4* SNP rs743554 is an independent prognostic marker of BC. However, this result should be taken with caution because complete clinical data were available only for 244 patients. Additionally, multivariate analysis is complicated by correlations between variables whereby causality cannot be inferred. Also the carriers of the AC and CC genotypes of the SNP rs2675 in the *ITGB5* gene tended to have hormone receptor-negative tumours. However, these associations were of borderline significance. No other associations between the studied SNPs and BC susceptibility or clinical outcome were observed.

So far, only a few polymorphisms in the integrin genes have been studied in relation to cancer risk and prognosis. Two SNPs in the *ITGA2* gene (Phe253Phe and Glu534Lys) have been associated with

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the risk of BC in one study, but not in another (20,21). The effect of the two SNPs on tumour grade or stage was also opposite in the two studies. Associations of the most commonly studied SNP in the integrin genes, Leu33Pro in the *ITGB3* gene, with BC have been contradictory (20–26). In two of the studies, the Pro allele has been associated with lymph node metastasis (20,24), whereas in the smallest study, the Leu allele was associated with positive lymph node status (21). The SNPs selected in our study have not been examined before for their effect on BC risk or on clinical outcome.

The association between the *ITGB4* and *ITGB5* SNPs and hormone receptor status may be explained through the fact that integrin-mediated signal transduction pathways have been shown to regulate ERα expression and protein levels in mouse mammary epithelial cells (27). Moreover, ECM proteins and their cellular receptors, such as integrins, may be critical for acquisition and loss of ovarian steroid function in normal and BC cells (28). On the other hand, ovarian steroids regulate ECM protein and integrin expression in the mammary gland. Changes in the composition of the ECM or in the expression of integrins, for example because of SNPs within the regulatory regions of the integrin genes, can lead to changes in the ECM receptor profiles of these cells, which, in turn, could alter the steroid hormone levels.

In addition to its effect on hormone receptor status, the *ITGB4* SNP was associated with BC-specific survival: the GA and AA genotype carriers of the SNP rs743554 had a worse survival than the non-carriers. The strongest association between the A allele carrier status and bad survival was observed in patients with more aggressive tumours (grade 3, lymph node metastasis-positive tumours and stages II–IV).

The integrin α6β4 containing the β4 subunit encoded by *ITGB4* has been shown to be over-expressed in many epithelial tumours, including tumours of the breast (29). It does not only promote tumour progression and spread but also promotes tumour cell survival by activating especially the PI3K/Akt pathway (29,30). Increased expression of the β4 integrin subunit has been associated with large tumour size and high-grade tumours (31). The importance of the β4 subunit on tumour cell survival has been demonstrated in mammary tumour cell lines in the absence of ovarian steroid signalling (30). Therefore, high α6β4 expression has also been correlated with poor prognosis (32), although a later study has observed only a non-significant trend for poor survival in patients with high β4 subunit expression (31).

The variant allele of the investigated SNP in the *ITGB4* gene may cause a loss of the binding site for the miRNA miR-34a. A global decrease of miRNA levels is often observed in human cancers, indicating the important role of miRNAs in tumour suppression (33). An increased expression of miR-34 in breast carcinoma compared with normal breast tissue has been observed (34). However, a number of miRNAs have been shown to be differentially expressed in subgroups of patients with ER+ versus ER− tumours (34–37). The list also includes several miRNAs, whose binding sites might be affected by the investigated SNPs (see supplementary Table III, available at *Carcinogenesis* Online). For the miR-34a, differential expression has been observed in tumours, which were characterized by their ER, PR and ErbB2 status (36). The miR-34 family is a direct transcriptional target of p53 and down-regulates a programme of genes promoting cell cycle progression (33). Moreover, in neuroblastoma, the miR-34a has shown to have tumour suppressor activity by inducing apoptosis through a caspase-dependent apoptotic pathway (38). By causing a loss of the miR-34a-binding site in the *ITGB4* gene, the SNP rs743554 may enhance the ability of integrin β4 to promote tumour cell growth, survival and invasion and thus partly explain the observed bad survival of the carriers of the variant allele.

The strengths of our study include the reasonable large sample size (749 cases, 1493 controls), the prospective nature of the blood sample collection, the long follow-up time (BC-specific follow-up up to 15 years) and the detailed clinical data. However, the long and variable follow-up time also brought some limitations to our study. Many of the cases diagnosed in the early 1990s did not have complete clinical data. This was especially true for the ER and PR status, where about half of the data were missing. The missing data of hormone receptor status also decreased the power to detect associations with genotypes. Consequently, although several SNPs in the integrin genes seemed to associate with hormone receptor status, only the associations with some genotypes in the *ITGB4* and *ITGB5* were statistically significant. As BC has a relatively good survival, >75% of the patients were alive at the end of the present follow-up period, the use of recurrence as an end point of survival would have been more robust than the use of death by BC. However, such data were not available.

Our results suggest a possible role for the investigated SNP rs743554 in the *ITGB4* gene in BC prognosis. As this SNP was associated with the hormone receptor status, it may influence tumour responsiveness to hormone therapy. The SNP rs743554 was also associated with a worse survival, especially in patients with more aggressive tumours. Since integrin-associated proteins are involved in all major signal transduction pathways regarding proliferation and survival, they are probably candidates for targeted therapies. The observed genetic variation may also cause inter-individual variation in response to integrin-targeted therapy. Additional studies are needed to confirm our data and to clarify further the role of integrins in BC prognosis.

Supplementary material

Supplementary Tables I–V can be found at http://carcin.oxfordjournals.org/

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