Integrin $\beta 3$ Leu33Pro polymorphism increases *BRCA1*-associated ovarian cancer risk

Anna Jakubowska, Jacek Gronwald, Janusz Menkiszak, Bohdan Górski, Tomasz Huzarski, Tomasz Byrski, Lutz Edler, Jan Lubiński, Rodney J Scott, Ute Hamann

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Integrins are heterodimeric transmembrane glycoproteins that function as key adhesion and cell signalling receptors. A functional polymorphism in the integrin $\beta 3$ subunit encoded by the *ITGB3* gene, Leu33Pro, has been shown to modify a variety of traits of $\beta 3$ -expressing cells. To analyse the role of this functional polymorphism in modifying *BRCA1*-associated ovarian and breast cancer risks, a case-control study was performed among Polish *BRCA1* mutation carriers including 319 breast cancer cases, 146 ovarian cancer cases and 290 controls unaffected by breast and ovarian cancer, in situ breast cancer or any other kind of cancer. Genotyping analysis was performed using PCR-based restriction fragment length polymorphism analysis. Odds ratios were calculated using univariate and multivariate logistic regression, taking into account a series of confounding variables, including the presence of related study subjects, that potentially could have biased any association. The results revealed that the *ITGB3*_Leu33Pro polymorphism was associated with a 2.5-fold increased risk of ovarian cancer, whereas no association with breast cancer risk was found. Thus, it appears that the *ITGB3*_Leu33Pro polymorphism may potentially increase the risk of ovarian cancer in Polish women with an inherited *BRCA1* mutation.

Integrins are a diverse family of heterodimeric transmembrane glycoproteins, each comprised of non-covalently linked α and β subunits. They influence growth factor signalling, cell survival, proliferation and migration via their function as surface adhesion and cell signalling receptors, and may play a major role in tumour behaviour and metastasis.^{1,2}

Evidence suggests that $\alpha v\beta 3$ and $\alpha IIb\beta 3$ integrins act as key players in cancer development and progression. Integrin $\alpha v\beta 3$ is expressed in several malignancies, and its expression correlates with tumour progression in melanomas, gliomas, ovarian and breast cancer.^{3–6} In particular, in breast cancer, it characterises the metastatic phenotype as it is upregulated in invasive tumours and distant metastases.⁷ Activation of $\alpha v\beta 3$ integrin promotes mechanical arrest of breast cancer cells during blood flow via an interaction with platelets.⁴ $\alpha IIb\beta 3$ integrin solely expressed in platelets and megakaryocytes is required for tumour-induced platelet aggregation and is involved in the adherence of tumour cells to endothelium, extravasation and angiogenesis, which are the key steps in the metastatic cascade.⁸

The integrin $\beta 3$ subunit is encoded by the *ITGB3* gene (Online Mendelian Inheritance in Man +173470). A previously reported substitution of T to C at codon 33 in the mature protein causes a leucine to proline exchange.⁹ This change is of structural and functional significance, in that it introduces a nick in the polypeptide chain just N-terminal to the $\beta 3$ hybrid domain that is involved in dimerisation with the αv and αIIb subunits. In platelets, it increases the binding of fibrinogen to $\alpha IIb\beta 3$,¹⁰

results in a decreased response to stimulation with thromboxane A_2 ,¹¹ decreases bleeding time,¹² enhances thrombin generation,¹³ increases the ability of platelets to aggregate¹⁴ and increases the activation of mitogen-activated protein kinases, resulting in increased tumour cell proliferation.¹⁵ Together, these changes in function make the *ITGB3* 33Pro allele a strong candidate as a cancer susceptibility variant.

Several studies have analysed the association of the *ITGB3*_Leu33Pro polymorphism with breast^{8,16–20} and ovarian cancer.^{16,21,22} However, no data on its influence on disease risks exist in women with an inherited predisposition to breast or ovarian cancer mediated by *BRCA1*.

In Poland, three common founder mutations in the *BRCA1* gene, 5382insC, 4153delA and 300 T→G, account for approximately 90% of all detected *BRCA1* mutations in families with breast-ovarian cancer.^{23,24} Because of the strong founder effect and the Polish population being relatively stable and ethnically homogeneous, it is ideal for association studies of risk-modifying genes not influenced by *BRCA1* allelic or ethnic variation. Given the plausible role of the *ITGB3*_Leu33Pro polymorphism in modification of *BRCA1*-associated breast and ovarian cancer risks, we undertook an analysis of the *ITGB3*_Leu33Pro polymorphism in Polish women harbouring one of the three Polish *BRCA1* founder mutations, including 319 breast cancer cases, 146 ovarian cancer cases and 290 asymptomatic controls.

METHODS

Study participants

The Hereditary Cancer Registry at the Pomeranian Medical University in Szczecin, Poland, contains clinical and epidemiological data collected from 1997 to 2002 from 1940 individuals carrying one of the three common Polish *BRCA1* founder mutations: 5382insC, 300 T→G and 4153delA. Mutation carriers were selected from families with at least one breast cancer diagnosed before 50 years of age or ovarian cancer at any age, or with a strong history of breast and/or ovarian cancer. A self-administered questionnaire was used to collect information on potential risk factors.

From the 1940 registered Polish *BRCA1* carriers, 755 female *BRCA1* mutation carriers from whom DNA samples were available were included in this study: 319 breast cancer cases, 146 ovarian cancer cases and 290 unaffected controls. Breast and ovarian cancer cases were diagnosed with invasive primary disease and had not undergone prophylactic mastectomy or adnexectomy (breast cancer cases) and prophylactic mastectomy, adnexectomy or tubal ligation (ovarian cancer cases) prior to the age of cancer diagnosis. Controls for breast cancer were unaffected by breast cancer, in situ breast carcinoma or any other type of cancer and had not undergone prophylactic mastectomy or adnexectomy, and controls for ovarian cancer

Abbreviation: BMI, body mass index

Table 1 Study groups, and median ages of cases at diagnosis of breast and ovarian cancer and of controls at the time of interview

No. of cases and controls*	Risk factor data available	Median age of cases at cancer diagnosis, years (range)	Median age of controls at the time of interview, years (range)
Breast cancer cases/controls			
319/290 (total)		41 (22–74)	44 (18–85)
232/225	Yes	41 (24–74)	44 (18–85)
87/65	No	39 (22–56)	48 (34–75)
Ovarian cancer cases/controls			
146/280 (total)		47 (25–75)	44 (18–85)
85/215	Yes	46 (25–71)	44 (18–85)
61/65	No	48 (27–75)	48 (34–75)

*Two hundred and eighty controls were used as controls for both breast cancer cases and ovarian cancer cases.

cases were unaffected by ovarian cancer or any other type of cancer and had not undergone prophylactic mastectomy, adnexectomy or tubal ligation. Table 1 presents the number of subjects in the various subgroups, and the median ages of cases at diagnosis of breast and ovarian cancer, and of controls at the time of interview.

Genetic analysis

Genomic DNA was isolated from peripheral blood leucocytes according to Lahiri and Schnabel.²⁵ Genotyping of the *ITGB3* 176 T→C (Leu33Pro) (rs5918) polymorphism in 755 *BRCA1* mutation carriers was performed by PCR and restriction fragment length polymorphism analysis, using *MspI* restriction enzyme.

The region with the *ITGB3* 176 T→C polymorphism was amplified using newly designed forward primer 5'-GCC TGC AGG AGG TGA AGA G-3' and Cy5-labelled reverse primer 5'-GCC TCA CTC ACT GGG AAC TC-3'. Amplified DNA fragments were digested with 2.5 U of *MspI* (New England Biolabs, Frankfurt, Germany) and fragments were separated by capillary gel electrophoresis on a CEQ 8000 fully automated DNA Analysis System (Beckmann, Krefeld, Germany). Separation of the CEQ DNA Size Standard-400 in each well allowed for CEQ automated sizing of Cy-5-labelled PCR products, with allele sizes of 168 bp for the T allele and 84 bp for the C allele. Each 96-well plate of genomic DNA contained multiple controls, including a water blank.

Genotyping was performed by laboratory personnel blinded to the case-control status. Accuracy and reproducibility of genotyping data were based on repeated analysis of 10% of randomly selected case and control samples. Genotypes were obtained for all 755 *BRCA1* mutation carriers, constituting a PCR concordance rate of 100%.

In addition to the examined polymorphism, we also identified the T to G nucleotide substitution changing a leucine to an arginine at codon 40 (rs36080296) in nine women (three breast cancer cases, five ovarian cancer cases and one control). The genotypes were confirmed by DNA sequencing. For the statistical analyses, we only evaluated the genotypes of the *ITGB3*_Leu33Pro polymorphism.

Statistical analysis

Risk estimates were calculated as odds ratios (ORs) with 95% CI using univariate and multivariate conditional logistic regression. Crude ORs (OR_{crude}) were calculated for 319 breast cancer cases and 290 controls, and for 146 ovarian cancer cases and 280 controls. For 232 breast cancer cases and 225 controls, and 85 ovarian cancer cases and 215 controls, we adjusted for potential breast/ovarian cancer risk factors, referred to as

adjusted ORs (OR_{adj}), by including age at menarche, age at first live birth (0, ≤24 and >24 years), parity, life time cumulative months of breastfeeding (≤12 and >12 months), oral contraceptive use (<5 and ≥5 years), smoking (0, <4 and ≥4 pack-years), body mass index (BMI; at age of breast cancer diagnosis for cases and at the corresponding age for controls), year of birth and *BRCA1* mutation in the multivariate logistic regression model. Leu/Pro and Pro/Pro genotypes were combined for regression analysis due to the low proportion of women with the Pro/Pro genotype (<3%) in patients and controls.

Age comparisons among carriers of different genotypes were performed using the Mann-Whitney U test. Exact 95% CIs were calculated for binomial probabilities. The statistical analyses were performed using the SAS/STAT(r) software, V.9.1, with the LOGISTIC procedure. In order to account for a potential bias due to the presence of relatives, both in the case and in the control groups, we also performed clustered multivariate logistic regression analysis using the SAS programme SURVEYLOGISTIC. Two-sided p values of ≤0.05 were considered as significant.

RESULTS

Breast and ovarian cancer cases and their corresponding controls were similar with respect to potential breast/ovarian cancer risk factors including year of birth, age at first live birth, age at menarche, BMI, parity, breastfeeding, smoking and *BRCA1* mutation.²⁶

Comparison of *ITGB3*_Leu33Pro allele and genotype frequencies among ovarian cancer cases and controls

Among *BRCA1* carriers, the prevalence of the Pro allele was significantly higher in cases compared with controls (57/292, 19%, 95% CI 15.1 to 24.5 vs 61/560, 11%, 95% CI 8.4 to 13.8) (Fisher's exact test, p<0.001). Table 2 shows the genotype distribution among ovarian cancer cases and controls. Carriers of the Pro allele had a significantly increased risk of developing ovarian cancer (OR_{adj} 2.51, 95% CI 1.30 to 4.84). After correction for related study subjects, a significant association remained (OR_{clustered} 1.97, 95% CI 1.53 to 2.53).

Among all ovarian cancer cases, the median ages at ovarian cancer diagnosis were similar between Pro allele carriers and non-carriers (46 years, range 25–75 years and 47 years, range 27–71 years, respectively; Mann-Whitney U test, p = 0.87).

Comparison of *ITGB3*_Leu33Pro allele and genotype frequencies among breast cancer cases and controls

No difference was observed in the allele and genotype frequencies between breast cancer cases and controls (table 2).

DISCUSSION

In this study, we investigated the effect of the *ITGB3*_Leu33Pro polymorphism on breast and ovarian cancer risk in female *BRCA1* mutation carriers from Poland. Although several studies on the effect of this polymorphism on sporadic breast and ovarian cancer risks^{8 16–18 20–22} and one study on familial breast cancer risk¹⁹ have been reported previously, data on its influence on hereditary breast and ovarian cancer risks are lacking.

In this study, we showed that women with *BRCA1* mutations who also carried the *ITGB3*_33Pro allele had an approximately 2.5-fold increased risk of ovarian cancer compared with women not carrying the Pro allele. This result remained after correcting for those study participants who were related to one another using a family cluster analysis, indicating that the observed modification of ovarian cancer risk can be attributed to the influence of the polymorphic *ITGB3* gene. Similar findings have

Table 2 Association of the *ITGB3* polymorphism with ovarian cancer risk and no association with breast cancer risk

Ovarian cancer									
Genotype/allele	Cases (n = 146) n (%)	Controls (n = 280) n (%)	OR _{crude} * (95% CI)	p Value	Cases (n = 85) n (%)	Controls (n = 215) n (%)	OR _{adj} † (95% CI)	p Value	OR _{clustered} ‡ (95% CI) p Value
<i>ITGB3</i> _Leu33Pro									
Leu/Leu	96 (66)	224 (80)	1.00 (reference)		57 (67)	172 (80)	1.00 (reference)		1.00 (reference)
Leu/Pro+Pro/Pro	50 (34)	56 (20)	2.08 (1.33 to 3.27)	0.002	28 (33)	43 (20)	2.51 (1.3 to 4.84)	0.006	1.97 (1.53 to 2.53) p<0.001
Pro	57 (19)	61 (11)			29 (17)	47 (11)			
Breast cancer									
Genotype/allele	Cases (n = 319) n (%)	Controls (n = 290) n (%)	OR _{crude} * (95% CI)	p Value	Cases (n = 232) n (%)	Controls (n = 225) n (%)	OR _{adj} † (95% CI)	p Value	OR _{clustered} ‡ (95% CI) p Value
<i>ITGB3</i> _Leu33Pro									
Leu/Leu	249 (78)	233 (80)	1.00 (reference)		184 (79)	181 (80)	1.00 (reference)		1.00 (reference)
Leu/Pro+Pro/Pro	70 (22)	57 (20)	1.15 (0.77 to 1.70)	0.62	48 (21)	44 (20)	1.12 (0.69 to 1.82)	0.63	1.14 (0.82 to 1.59) 0.43
Pro	76 (12)	62 (11)			49 (11)	48 (11)			

*OR_{crude}, crude odds ratio.†OR_{adj}, odds ratio adjusted for year of birth, age of first live birth, age at menarche, body mass index, parity, breastfeeding, oral contraceptive use, smoking and *BRCA1* mutation.‡OR_{clustered}, odds ratio on correction for family cluster.

been reported for sporadic ovarian cancer in two prospective population-based studies of 4291 participants including 28 cases (hazard ratio 3.9, 95% CI 1.1 to 13) and 9242 participants including 36 cases (risk ratio 4.7, 95% CI 1.6 to 14), and in one case-control study with 463 cases and 3543 controls (OR 1.6, 95% CI 1.0 to 2.6) conducted in Denmark.^{16,22} In another German population-based case-control study of 240 ovarian cancer cases and 426 controls, no increase in risk among homozygote carriers of the 33Pro allele was reported (OR 1.23, 95% CI 0.41 to 3.69). However, a higher proportion of 33Pro carriers was found among patients with ovarian cancer with adverse prognostic markers than those without, suggesting that the *ITGB3*_Leu33Pro polymorphism influences the metastatic spread and the malignant potential of ovarian cancer.²¹

The molecular mechanism behind the increased ovarian cancer risk in *ITGB3*_33Pro carriers is unknown. However, according to the functional data of this polymorphism, one would expect the *ITGB3*_33Pro allele rather than the *ITGB3*_33Leu allele to increase cancer risk and/or metastatic potential. There is evidence that the *ITGB3*_33Pro allele increases the adhesive properties of tumour cells¹⁴ and enhances the activation of mitogen-activated protein kinase pathways,¹⁵ crucial for the malignant potential of cancer cells.²⁷

The effect of the *ITGB3*_Leu33Pro polymorphism on ovarian cancer risk could not be extended to differences in the age at diagnosis of disease in patients with the *ITGB3*_Pro/Pro genotype and in those with the *ITGB3*_Leu/Leu+Leu/Pro genotypes, suggesting that other factors may influence the effects of this change with respect to the age of disease onset.

The *ITGB3*_Leu33Pro polymorphism was not associated with *BRCA1*-associated breast cancer in this study. The most likely explanation for the difference in effect between patients with ovarian cancer and those with breast cancer within our study set is in the underlying disease mechanisms involved in ovarian cancer compared with breast cancer. At this stage, it remains to be resolved what these differences are.

In our study, risks were adjusted for known and putative breast and ovarian cancer risk factors including age at menarche, age of first live birth, parity, breastfeeding, oral contraceptive use, smoking status, BMI, year of birth and *BRCA1* mutation. We refrained from adjusting for hormone replacement therapy due to the small number of users of hormone replacement therapy. These findings imply that the

observed modification of ovarian cancer risk can be attributed to the influence of the polymorphic *ITGB3* gene.

Our study benefits from having a sample of reasonable size required for these kinds of studies. The study population consisted of women who harboured one of the three common Polish founder mutations in a gene known to confer a high risk of disease and a group of women who also harbour one of these mutations but have not developed malignancy. This study highlights the potential benefits of investigating modifiers of breast and ovarian cancer risk in populations with little *BRCA1* allelic and ethnic variation.

Some limitations of the present study should be taken into account. A general selection bias might have occurred due to the use of study participants from a registry. Further, due to the inclusion of incident and prevalent cases in the Polish registry, the presence of prevalent cases among our study participants might have led to a survival bias, which is a general limitation of this type of retrospective study. The frequency of *BRCA1* mutation carriers in the Polish population is nearly 0.5%,²⁸ making a recruitment of study participants from the population unrealistic for this kind of study. Therefore, the use of a registry seems to be the only acceptable way to perform the study. The effect of this change on proliferation and adhesive properties of tumour cells suggests that the *ITGB3*_Leu33Pro polymorphism may modify disease risk itself and is not due to any other sequence change in a regulatory region of the gene or in a nearby gene that is in linkage disequilibrium with this polymorphism.

At present, clinical genetic testing for breast and ovarian cancer is limited to genes with highly penetrant, rare cancer predisposition alleles such as *BRCA1* and *BRCA2*. Genetic testing for weakly penetrant alleles is not done due to the low increased disease risk and the poor predictive value of the genotype. However, after the identification of enough low-penetrant alleles accounting for a substantial increased risk, additional genotyping of a panel of such loci may allow a more accurate risk assessment in women with an inherited predisposition.

Current preventive strategies for ovarian cancer in *BRCA* mutation carriers include annual pelvic examination and transvaginal ultrasound combined with serum carbohydrate antigen 125 assessment, prophylactic bilateral oophorectomy²⁹ and bilateral salpingo-oophorectomy.³⁰ Genotyping for

ITGB3_Leu33Pro polymorphism may be useful before ovarian cancer diagnosis and may also assist in the future in drug selection at the time of diagnosis. Blockage of $\beta 3$ -mediated signal transduction by an anti- αv -antibody has been shown to result in a reduced growth rate of ovarian cancer cell lines in vitro.³¹

In conclusion, we have shown that the ITGB3_33Pro allele of the $\beta 3$ integrin component seems to be associated with an increased risk of ovarian cancer in Polish BRCA1 mutation carriers. If this association is confirmed in other larger studies, determination of the ITGB3 status and other still to be identified low-penetrance alleles may provide an additional guideline for risk assessment, genetic counselling and management of such women.

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Authors' affiliations

Anna Jakubowska, Jacek Gronwald, Bohdan Górski, Tomasz Huzarski, Tomasz Byrski, Jan Lubiński, Department of Genetics and Pathology, International Hereditary Cancer Center, Pomeranian Medical University, Szczecin, ul Polabska, Poland

Janusz Menkiszak, Department of Surgical Gynecology and Gynecological Oncology of Adults and Adolescents, Pomeranian Medical University, ul Powstancow Wlkp, Szczecin, Poland

Anna Jakubowska, Ute Hamann, Division of Molecular Genome Analysis, Molecular Genetics of Breast Cancer, German Cancer Research Center, Heidelberg, Germany

Lutz Edler, Central Unit Biostatistics, German Cancer Research Center, Heidelberg, Germany

Rodney J Scott, Discipline of Medical Genetics, School of Biomedical Sciences, University of Newcastle, and the Hunter Medical Research Institute, New Lambton, New South Wales, Australia

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Correspondence to: Prof U Hamann, German Cancer Research Center, Division of Molecular Genome Analysis, Molecular Genetics of Breast Cancer, B055, Im Neuenheimer Feld 580, 69120 Heidelberg, Germany; u.hamann@dkfz-heidelberg.de

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