

Increased risk of ovarian cancer in integrin β_3 Leu33Pro homozygotes

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Abstract

We previously demonstrated that integrin β_3 Leu33Pro homozygotes have an increased risk of cancer, possibly most pronounced for ovarian cancer. We now test the latter hypothesis in case-control and prospective studies. We genotyped 463 Danish women with ovarian cancer, and 4291 women from the Danish general population. Calculation of odds ratios by conditional logistic regression was performed in the case-control study ($n=463+3543$), and of ovarian cancer incidence, log-rank statistics and hazard ratios by Cox regression in the prospective study ($n=4291$) with 9.5-year follow-up. In the case-control study matched for age and marital status, the odds ratio for ovarian cancer in homozygotes versus non-carriers was 1.6 (95% confidence interval: 1.0–2.6). In the prospective study with 28 incident ovarian cancers, non-carriers and homozygotes had incidences of 7 (4–11) and 30 (10–92) per 10 000 person-years (log-rank $P=0.02$). The age-adjusted hazard ratio for ovarian cancer in homozygotes versus non-carriers was 3.9 (1.1–13). Risk of ovarian cancer did not differ between heterozygotes and non-carriers in either study. Integrin β_3 Leu33Pro homozygotes have an increased risk of ovarian cancer.

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Introduction

It is thought that genetic factors explain roughly 22% (95% confidence interval: 0–41%) of all ovarian cancer (Lichtenstein *et al.* 2000). Mutations in *BRCA1* and *BRCA2* and in the genes involved in hereditary non-polyposis colorectal cancer have been identified as causes of familial aggregation of ovarian cancer; however, these mutations explain only about 40% of the familial cases (Gayther *et al.* 1999) and only 2–5% of all ovarian cancer (Pharoah & Ponder 2002). Therefore, other, presumably low-penetrant ovarian cancer susceptibility loci probably constitute the main bulk of the genetic contribution to ovarian cancer.

In a recent large prospective study of the general population, we demonstrated that germline integrin β_3 Leu33Pro homozygotes have an increased risk of cancer, which in a post hoc explorative analysis

appeared to be most pronounced for ovarian cancer (Bojesen *et al.* 2003). Biologically this seems plausible for the following reasons:

1. β_3 integrin expression is higher in ovarian cancer than in benign ovarian tumours (Liapis *et al.* 1997).
2. In ovarian cancer cells, β_3 integrins interact with their ligands vitronectin (Hapke *et al.* 2003) and periostin (Gillan *et al.* 2002), altering the intracellular levels of factors essential for the adhesive, migratory and proliferative phenotype of human ovarian cancer cells (Rangaswami *et al.* 2004).
3. A third ligand of β_3 integrins is osteopontin (Panda *et al.* 1997), recently identified as a potential diagnostic marker for ovarian cancer (Kim *et al.* 2002); upon binding to β_3 integrin, osteopontin releases migratory responses,

increased in cells expressing the Pro33 version of β_3 integrin compared with the Leu33 version (Sajid *et al.* 2002).

4. The Pro33 versus Leu33 version of β_3 integrins also enhances integrin-mediated activation of mitogen-activated, protein-kinase pathways (Vijayan *et al.* 2003), crucial for the malignant potential of cancer cells (Johnson & Lapadat 2002).

We therefore directly tested the hypothesis that integrin β_3 Leu33Pro homozygosity is associated with an increased risk of ovarian cancer.

First, we tested the hypothesis in a case-control study of 463 Danish women with ovarian cancer compared with 3543 Danish control women from the general population matched for age and marital status. In this study, we also investigated whether increased risk of ovarian cancer among integrin β_3 Leu33Pro homozygotes depends on parity and menopausal status. Finally, we re-examined our prospective study of 4291 women from the general population (Bojesen *et al.* 2003), but in a version improved in several ways, now extending the follow-up for more than 3 years until the end of 2002.

Materials and methods

Case-control study

The subjects were 463 women with invasive ovarian cancer from the population-based MALOVA (*MALig-nant OVarian*) study (Høgdall *et al.* 2003a,b, Glud *et al.* 2004). The MALOVA study was a multi-disciplinary Danish study on ovarian cancer, covering epidemiology, biochemistry and molecular biology. From 1 December 1994 to 31 May 1999, at the gynaecological departments in a certain area of Denmark (municipality and county of Copenhagen, municipality of Frederiksberg, and counties of Roskilde, Western Zealand, Funen, and Southern and Northern Jutland), all available women aged 35–79 years and diagnosed with an ovarian tumour were included. A blood sample for DNA analyses was drawn prior to surgery, and after the operation patients were interviewed concerning medical, marital, menopausal and reproductive history. According to the protocol, women with borderline and benign ovarian tumours were also enrolled in the MALOVA study, but for the present study, only women with invasive ovarian cancer were included. The control group of the case-control study comprised women from the Danish general population in the prospective study who did not develop ovarian cancer (see below),

matched with the MALOVA women on age at blood sampling and on marital status ($n = 3543$).

Prospective study

In the 1991–4 examination of the Copenhagen City Heart Study, 5111 women from the Danish general population of Copenhagen participated. More than 99% were white of Danish descent. Questionnaire information included medical, marital, menopausal and reproductive history. A blood sample for DNA analyses was drawn. Women answering ‘yes’ to the question, ‘Did your menstruation cease due to an operation?’ ($n = 806$), were excluded, because we could not be sure whether they had had their ovaries removed and consequently no longer were at risk of developing ovarian cancer. An additional 14 women with a diagnosis of ovarian cancer occurring outside the follow-up period were excluded, leaving 4291 participants for the present prospective study. Diagnoses of ovarian cancer (World Health Organisation (WHO) *International Classification of Diseases* (7th edn), codes 175.0, 175.1, 175.2, 175.3, 175.5, 176.9, 375.0, 475.0), were obtained from the Danish National Cancer Registry covering the period from 1 January 1946 until 31 December 2002. Follow-up started at the time of blood sampling and ended at death or 31 December 2002, whichever came first.

The re-examination of the prospective study included in the present paper has been improved in the following ways in addition to the extended follow-up:

1. To reduce any bias due to genotype-dependent survival, follow-up now starts at the day of blood sampling in 1991–4, rather than at study entry in 1976–8.
2. Our previous explorative study (Bojesen *et al.* 2003) used the classification ‘ovarian cancer etc.’ including cancers of the fallopian tube, vagina and vulva according to WHO (Bray *et al.* 2002), but in the present study only invasive ovarian cancers were included.
3. Risk of ovarian cancer was examined as a function of age, and not as a function of follow-up time as in the previous study (Bojesen *et al.* 2003).
4. Women who might have had their ovaries removed prior to entry were excluded ($n = 806$).

Ethics approval

The ethics committee of Copenhagen and Frederiksberg approved the prospective study and

the MALOVA studies (100.2039/91 and KF01-384/95). The studies were also approved by Herlev University Hospital.

Genotyping

Participants were genotyped as described earlier (Zimrin *et al.* 1990) by genomic DNA isolated from leukocytes from peripheral blood. In short, the Leu33Pro polymorphism is a T→C substitution in exon 3 at position 176 in the integrin β_3 gene (GenBank accession no. NM_000212.1), which introduces an MspI recognition site. The assay also included a second MspI recognition site always cleaved, which served as a control site for the digestion reaction. A 268 bp fragment of exon 3 was amplified from genomic DNA with flanking intronic primers, cleaved with MspI, run on a 3% agarose gel and visualized by staining with ethidium bromide. Genotypes were determined independently by an experienced laboratory technician and an author (S E B). Control sequencing confirmed genotype in selected samples.

Statistical analysis

We used the statistical software package STATA (2004). Two-sided probability values of <0.05 were considered significant. Mann–Whitney *U* test and Pearson's chi-square test were used. Reference population was Leu33Pro non-carriers. Heterozygotes and homozygotes were each compared with reference.

In the case-control study, genotype specific risk of ovarian cancer was expressed as an odds ratio, using conditional logistic regression. We matched cases with controls for marital status (ever/never married or living with a partner) and age at blood sampling in 2-year strata. Marital status as a matching parameter was chosen to reduce any distortion due to the higher frequency of single-living women without children among the control population from Copenhagen city

than among the case population from the whole country. Controls in strata without cases were excluded from the analyses. This resulted in 40 strata with an average of 7.7 controls per case. This model was run unadjusted and adjusted for parity and menopausal status. Interaction between genotype (homozygosity vs non-carrier) and parity/menopausal status was tested by introducing two-factor interaction terms in models already including genotype and parity/menopausal status.

In the prospective study, we tested differences in ovarian cancer incidence as a function of left truncated age between genotypes by log-rank statistics. Genotype specific risk of ovarian cancer was expressed as a hazard ratio, using a Cox regression model with left truncated age as time scale. As age is the underlying time variable in this model, age is automatically adjusted for. The multifactorial adjusted model also included parity (nulliparous vs parous) and menopausal status (pre- vs postmenopausal) at the examination date.

Results

Characteristics of participants are shown in Table 1. In women from the general population ($n = 4291$), 3018 (70%) were non-carriers, 1157 (27%) heterozygotes and 116 (3%) homozygotes. This genotype distribution was in Hardy–Weinberg equilibrium ($P = 0.69$). Neither in the case-control study nor in the prospective study did age or parous status of cases differ from that of controls; however, in both studies, more cases than controls were postmenopausal ($P = 0.006$ and $P = 0.03$).

In the matched case-control study, the odds ratio for ovarian cancer in homozygotes versus non-carriers was 1.6 (1.0–2.6) (Table 2). Adjustment for parity and menopausal status produced an equivalent odds ratio of 1.6 (0.9–2.7); because these covariates were unknown for 93 cases, statistical power was reduced

Table 1 Characteristics of participants

	Case-control study ($n=4006$)			Prospective study ($n=4291$)		
	Ovarian cancer cases	Controls	<i>P</i> value	Ovarian cancer cases	Other participants	<i>P</i> value
Number	463	3543		28	4263	
Age (years)	61 (38–78)	61 (35–79)	0.36	63 (37–77)	60 (26–83)	0.15
Nulliparous (%)	18 (14–22)	18 (17–20)	0.79	25 (8–42)	16 (15–17)	0.72
Postmenopausal (%)	83 (79–87)	77 (75–78)	0.006	86 (72–100)	66 (65–68)	0.03

Values represent median (2.5–97.5 percentiles) or frequencies (95% binomial confidence interval).

Ovarian cancer cases versus controls/other participants on Mann–Whitney *U* test or Pearson's chi-square test.

Cases and controls were matched on age and marital status in the case-control study.

Table 2 Risk of ovarian cancer by integrin β_3 Leu33Pro genotype

			Non-carriers 33Leu/Leu	Heterozygotes 33Leu/Pro	Homozygotes 33Pro/Pro
Case-control study		Cases	Controls	Odds ratio (95% confidence interval)	
Matched for age and marital status	463	3543	1.0	1.1 (0.9–1.4)	1.6 (1.0–2.6)
Matched for age and marital status and multi-factorially adjusted*	370	3528	1.0	1.0 (0.8–1.3)	1.6 (0.9–2.7)
Prospective study		Events	Participants	Hazard ratio (95% confidence interval)	
Adjustment for age	28	4291	1.0	0.8 (0.3–2.0)	3.9 (1.1–13)
Multifactorially adjusted*	28	4291	1.0	0.8 (0.3–2.0)	3.9 (1.1–13)

*Additionally adjusted for parity (nulliparous vs parous) and menopausal status (pre- vs postmenopausal).

in this multifactorial adjusted model versus the matched only model. Risk of ovarian cancer in heterozygotes did not differ from that in non-carriers.

We next looked for context-dependent associations between genotype and risk of ovarian cancer in the case-control study (Table 3). Odds ratios for ovarian cancer in homozygous versus non-carriers were 4.6 (1.6–13) and 1.2 (0.6–2.4) among nulliparous and parous women respectively; genotype by parity interaction was borderline significant ($P=0.06$, not adjusted for multiple comparisons). Menopausal status did not influence risk of ovarian cancer for homozygotes versus non-carriers (Table 3); genotype by menopausal status did not interact in relation to risk of ovarian cancer ($P=0.84$).

In the prospective study, the total follow-up time was 38 642 person-years with a median follow-up of 9.5 years. Twenty-eight incident ovarian cancer events were recorded corresponding to an incidence of 7 (95% confidence interval: 5–10) per 10 000 person-years. Non-carriers, heterozygotes and homozygotes had incidences of 7 (4–11), 6 (3–13) and 30 (10–92) per 10 000 person-years respectively. The incidence was higher in homozygotes than in non-carriers, but similar

in heterozygotes and non-carriers (log-rank $P=0.02$ and $P=0.61$ respectively). Age-adjusted and multi-factorially adjusted hazard ratios for ovarian cancer in 33Pro/Pro homozygotes versus non-carriers were 3.9 (1.1–13) and 3.9 (1.1–13) (Table 2). Due to lack of statistical power, it was not possible to look for context-dependent associations in the prospective study. Risk of ovarian cancer in heterozygotes did not differ from that in non-carriers.

Discussion

In women from the Danish general population, we observed an increased risk of ovarian cancer in integrin β_3 Leu33Pro homozygotes versus non-carriers in both case-control and prospective study designs. The present finding of increased risk of ovarian cancer in Leu33Pro homozygotes agrees with our previous results from an explorative epidemiological study (Bojesen *et al.* 2003). In that study, the primary hypothesis was that Leu33Pro homozygosity influences risk of all cancer, as it did, while risk of site-specific cancers was examined as post hoc analyses. Although the risk of ovarian cancer, breast cancer and melanoma

Table 3 Risk of ovarian cancer by integrin β_3 Leu33Pro genotype stratified for other risk factors in the case-control study

	Pro33 allele frequency (%)	Non-carriers 33Leu/Leu		Heterozygotes 33Leu/Pro		Homozygotes 33Pro/Pro	
	Cases/ Controls	Cases/ Controls	Odds ratio	Cases/ Controls	Odds ratio (95%CI)	Cases/ Controls	Odds ratio (95%CI)
Nulliparous	24.2/15.9	40/458	1.0	20/176	1.2 (0.7–2.2)	6/15	4.6 (1.6–13)
Parous	16.4/16.4	214/2016	1.0	80/781	0.9 (0.7–1.2)	10/83	1.2 (0.6–2.3)
Premenopausal	15.1/15.5	46/591	1.0	15/213	0.7 (0.4–1.4)	2/21	1.3 (0.3–5.8)
Postmenopausal	18.4/16.6	208/1886	1.0	85/743	1.1 (0.8–1.4)	14/77	1.5 (0.8–2.8)

Cases and controls were matched for age and marital status. CI=confidence interval.

For 56 non-carrier cases and 9 and 6 non-carrier controls, parity and menopausal status were unknown.

For 34 heterozygous cases and 4 and 5 heterozygous controls, parity and menopausal status were unknown.

For 3 homozygous cases and 1 homozygous control, parity and menopausal status were unknown.

all appeared to be increased in Leu33Pro homozygotes in those analyses (Bojesen *et al.* 2003), after correction for multiple comparison, only the increased risk of ovarian cancer remained significant.

In a population-based, age-matched case-control study of 240 ovarian cancer case patients versus 426 age-matched control subjects, Wang-Gohrke and Chang-Claude (2005) observed an odds ratio in integrin β_3 Leu33Pro homozygotes versus non-carrier women of 1.2 (0.4–3.7), which at first hand would appear to contrast with our results of an equivalent odds ratio in the case-control study of 1.6 (1.0–2.6) and an equivalent hazard ratio in the prospective study of 3.9 (1.1–13). In contrast to our study, they included borderline tumours, adjusted for first-degree family history of ovarian cancer, and had less statistical power than our case-control study of 463 cases and 3543 controls, so we cannot exclude that their results are compatible with ours (Bojesen *et al.* 2005). Importantly, however, because the two case-control studies had lower risk estimates than our prospective study, it is likely that the magnitude of the association between integrin β_3 Leu33Pro homozygosity and the risk of ovarian cancer is less than that originally observed in our exploratory analysis (Bojesen *et al.* 2003). Interestingly, Wang-Gohrke and Chang-Claude also observed a higher proportion of Leu33Pro carriers among ovarian cancer patients with adverse prognostic markers than those without, suggesting that the integrin β_3 Leu33Pro polymorphism is involved in the metastasis of, and therefore is an indicator of, the malignant potential of ovarian cancer.

Several differently designed studies on ovarian cancer and integrins suggest that this association between integrin β_3 Leu33Pro polymorphism and ovarian cancer incidence and/or prognosis may not be a coincidence:

1. In contrast to normal ovaries, expression of β_3 integrins is a common feature of epithelial ovarian cancer (Bridges *et al.* 1995, Cannistra *et al.* 1995), where they promote proliferation and survival of ovarian cancer cells (Cruet-Hennequart *et al.* 2003).
2. Integrins are crucial to cell invasion (Hood & Cheresch 2002), and inhibition of integrin β_3 function inhibits migration of human ovarian tumour cells (Szaniawska *et al.* 2001).
3. The Pro33 versus Leu33 allele enhances the activation of intracellular signalling pathways relevant to cancer development (Vijayan *et al.* 2003) as well as increases adhesive properties of

the cells (Feng *et al.* 1999, Vijayan *et al.* 2000, Bennett *et al.* 2001).

In view of all this evidence, we therefore speculate that the adhesive properties of the Pro33 version of β_3 integrins increase the probability that premalignant ovarian cells with this genotype have an advantage with regard to migration, survival and/or adhesion to the extracellular matrix, and thereby facilitate neoplastic growth and/or metastasis.

Our studies have some limitations. First, the rarity of 33Pro/Pro homozygosity status limits our statistical power to explore interaction between homozygosity and parity and/or menopausal status in detail. This is certainly the case in the prospective study with only three homozygotes with incident ovarian cancer. In the case-control study, we observed a markedly increased risk of 4.6 (1.6–13) for homozygosity versus non-carriers among nulliparous women, but as this finding included only 6 cases and 15 controls among homozygotes, we cannot exclude that the magnitude of this risk estimate arose by chance.

Second, even though we adjusted for both parity and menopausal status, in the prospective study and in the controls of the case-controls study, we do not have information on some other commonly recognized risk factors for ovarian cancer like duration of oral contraceptive use, duration of hormone replacement therapy, and familial history of ovarian cancer. This is an important limitation since it limits our ability to make inferences of the risk according to genotype in different contexts. However, because of Mendelian randomization (Smith & Ebrahim 2004), it is unlikely that use of oral contraceptives or hormone replacement is associated with genotype. Therefore, it is not likely that adjustment for these factors would significantly change our results on genotype and risk of ovarian cancer. Our lack of information on familial history of ovarian cancer limits our ability to infer the overall genetic make-up of cases and controls. On the other hand, adjusting for family history in studies examining association between genetic factors and disease risk might eliminate the very association examined, because genetic variation is inherited.

Third, we used a case-control analysis and a cohort analysis to test the hypothesis of a previously observed association between integrin β_3 Leu33Pro homozygosity and ovarian cancer risk. These two analyses used practically the same controls, since the controls were drawn from our original Danish cohort study. The cohort analysis was based on an extended follow-up of our original Danish cohort. Therefore, these two

analyses cannot be strictly considered a totally independent testing of the hypothesis developed in the Danish cohort study. Nevertheless, independent population-based cases were employed for the case-control analysis, and the results suggest that integrin β_3 Leu33Pro homozygosity is associated with ovarian cancer risk. However, the magnitude of the association needs to be estimated and confirmed in other population samples.

Despite the fact that ovarian cancer is one of the leading causes of cancer death in women (Ferlay *et al.* 2004), our current capability of predicting risk of ovarian cancer is mainly limited to estimates based on age, family history of ovarian cancer, nulliparity, long menstrual life, no use of oral contraceptive pills and use of hormone replacement therapy (Glud *et al.* 2004). Identification of specific genetic variations in genes, such as *TP53*, progesterone and androgen receptors, *STK15*, human leucocyte antigens (Agorastos *et al.* 2004, Agoulnik *et al.* 2004, Dicioccio *et al.* 2004, Monos *et al.* 2005, Terry *et al.* 2005) or integrin β_3 , associated with increased risk of ovarian cancer in the general population may contribute to risk-profiling of women with increased risk of ovarian cancer for further preventive procedures. Population-attributable risk (Levin 1953) for ovarian cancer of β_3 Leu33Pro homozygosity was estimated to be 8% in the prospective study, with hazard rates of 3.9% and 2% estimated in the case-control study with an odds ratio of 1.6. As other studies have also shown that the frequency of β_3 Leu33Pro homozygosity is 2–3% in Caucasian populations in Germany, the USA, France and Italy (Di Castelnuovo *et al.* 2001), our results are likely to apply to other women in the affluent world. Genotyping for integrin β_3 Leu33Pro might be useful not only before ovarian cancer diagnosis, but also for prognostic purposes, perhaps in combination with measurement of its ligand osteopontin (Brakora *et al.* 2004, Coppola *et al.* 2004, Schorge *et al.* 2004). Finally, in the future, genotyping at the time of diagnosis might also assist in drug selection: blockade of integrin β_3 -mediated signal transduction inhibits growth of human ovarian cancer cell lines (Szaniawska *et al.* 2001, Cruet-Hennequart *et al.* 2003), and interaction between integrin β_3 and its agonists and antagonists depends on Leu33Pro genotype (Michelson *et al.* 2000, Boncler *et al.* 2002, Wheeler *et al.* 2002, Angiolillo *et al.* 2004).

In conclusion, in a case-control study and a prospective study, we demonstrated that women homozygous for the Leu33Pro polymorphism of the β_3 integrin subunit have an increased risk of ovarian cancer.

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