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ORIGINAL RESEARCH ARTICLE

An Analysis of Polymorphisms Within the Wnt Signaling Pathway in Relation to Ovarian Cancer Risk in a Polish Population

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Abstract
Background and Objective The Wnt/β-catenin signaling pathway has been considered to be a factor in the development and progression of ovarian cancer.
Methods All patients with ovarian cancer and controls were tested for *BRCA1* mutations (5382incC, C61G, 4153delA) with HybProbe assays and for *BRCA2* mutation (5946delT) using high-resolution melting curve analysis (HRM). Mutation carriers were excluded from the association analysis. We studied nine single nucleotide polymorphisms (SNPs) located in *CTNNB1* (β-catenin) [rs4533622, rs2953], *APC* (rs11954856, rs351771, rs459552), and *AXIN2* (rs4074947, rs7224837, rs3923087, rs2240308) in women with ovarian cancer without *BRCA1/BRCA2* mutations ($n = 228$) and controls ($n = 282$). Genotyping of *CTNNB1* rs4533622, rs2953, *APC* rs351771, *AXIN2* rs4074947, rs3923087, and rs2240308 was performed by HRM, while that of *APC* rs11954856, rs459552 and *AXIN2* rs7224837 was conducted by PCR followed by the appropriate restriction enzyme digestion [PCR–restriction fragment length polymorphism (PCR-RFLP)].
Results The most common *BRCA1/BRCA2* mutations were identified in 30 patients with ovarian cancer. These mutations were not found in controls. The lowest p values of the trend test (p_{trend}) were observed for the *APC* rs351771 and rs11954856 SNPs in patients with ovarian cancer ($p_{trend} = 0.006$ and $p_{trend} = 0.007$, respectively). Using a dominant inheritance model, we found that the *APC* rs11954856 SNP is associated with an increased risk of ovarian cancer development [odds ratio = 2.034 (95 % CI 1.302–3.178); $p = 0.002$]. We also observed significant allelic differences for the *APC* rs351771 SNP between patients and controls ($p = 0.006$).
Conclusion Our study demonstrated significantly increased *APC* rs11954856 and rs351771 SNP frequencies in Polish women with ovarian cancer.

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1 Introduction

Ovarian cancer has the highest mortality among gynecological malignancies in Europe and the USA [1, 2]. Several factors, including genetic background, are known to increase the risk of ovarian cancer development [3]. To the well-recognized hereditary factors of ovarian cancer belong some of high-penetrance genes: *BRCA1* (3–6 %), *BRCA2* (1–3 %), and HNPCC DNA mismatch repair genes (1–2 %) [3–5]. However, these gene mutations explain less than 40 % of the familial predisposition to ovarian cancer [6]. These data indicate that the missing heritability can be elucidated by a multigenic disease model with contribution of moderate and low-penetrance risk genes to ovarian malignancies [6].

Several intracellular pathways have been recognized in ovarian carcinogenesis [7]. Among them, the Wnt/β-catenin/T-cell factor (TCF)/lymphoid enhancer factor (Lef) signaling pathway has been considered to be essential for the growth and progression of ovarian cancers [8]. The Wnt/β-catenin signaling pathway regulates a variety of elementary cellular functions, such as proliferation, polarity, adhesion, and motility during development, differentiation, and adult tissue homeostasis [9]. This pathway is also essential for normal ovarian development, and different components of the canonical Wnt signaling pathway are presented in the ovary [10, 11]. Wnt stimulation leads to a cytoplasmic accumulation of β-catenin that is subsequently translocated to the nucleus, where it interacts with the TCF family and induces the transcription of Wnt target genes [12]. The absence of Wnt stimulation results in β-catenin phosphorylation by components of the cytosol multi-protein degradation complex and its subsequent proteosomal degradation [12]. This multi-protein degradation complex contains adenomatous polyposis coli (APC), conductin (AXIN1 and AXIN2), glycogen synthase kinase-3β (GSK3β), and casein kinase 1 (CK1) [12].

The components of the Wnt/β-catenin/TCF/Lef signaling pathway have been reported to be up-regulated in various cancer types, including ovarian cancers [13, 14]. Moreover, many studies have demonstrated that the presence of mutations or polymorphisms in *CTNNB1*, *APC*, and *AXIN2* can lead to aberrant activation of Wnt/β-catenin signaling at the onset of various types of malignancies, including ovarian cancer [15–31]. In addition to these findings, inactivation of the *APC* gene in mice leads to the deregulation of Wnt/β-catenin signaling and the formation of adenocarcinomas that are morphologically similar to human ovarian endometrioid adenocarcinoma [32]. Therefore, we selected nine single nucleotide polymorphisms (SNPs) of *CTNNB1*, *APC*, and *AXIN2* located in distinct blocks of linkage disequilibrium (LD) in order to study whether these SNPs can be genetic risk factors of ovarian cancer (Online Resource Supplemental Table 1 and Supplemental Fig. 1A, Fig. 1B and Fig. 1C.). Selected SNPs were tested in the group of ovarian cancer patients and controls who did not carry the most common mutations of the *BRCA1*/*BRCA2* genes.

2 Material and Methods

2.1 Patients and Controls

The patients include 258 women with histologically determined ovarian carcinoma according to the International Federation of Gynecology and Obstetrics (FIGO). They were inducted into the study from the University Hospital, Clinic of Gynecological Surgery and Chair of Gynecologic Oncology at Poznań University of Medical Sciences (Poznań, Poland). Histopathological classification, including the stage, grade, and tumor type, was performed by an experienced pathologist (Table 1). The control group included 282 unrelated healthy female volunteers who were matched by age to the female cancer patients. Controls were selected during medical examination at the University Hospital, Clinic of Gynecological Surgery at Poznań University of Medical Sciences (Table 1). Written informed consent was obtained from all participating individuals. The procedures of the study were approved by the Local Ethical Committee of Poznań University of Medical Sciences. All women with ovarian cancer and controls were Caucasian from the Wielkopolska area of Poland.

2.2 Genotyping

Genomic DNA was isolated from peripheral blood leukocytes by salt extraction.

All patients and controls with ovarian cancer were tested for the three most common *BRCA1* mutations (5382incC, C61G, 4153delA) affecting the Polish population using the LightCycler® 480 system with HybProbe assays (Roche, Indianapolis, IN, USA). In addition, the patient group was

Table 1 Clinical characteristics of ovarian cancer patients and healthy controls

Characteristic	Patients ($n = 258$)	Controls ($n = 282$)
Mean age (years) ± SD	58.4 ± 9.7	57.4 ± 7.5
Histological grade		
G1	83 (32.2 %)	
G2	85 (32.9 %)	
G3	90 (34.9 %)	
Gx	0 (0.0 %)	
Clinical stage		
I	96 (37.2 %)	
II	40 (15.5 %)	
III	88 (34.1 %)	
IV	34 (13.2 %)	
Histological type		
Serous	90 (34.9 %)	
Mucinous	30 (11.6 %)	
Endometrioid	48 (18.6 %)	
Clear cell	24 (9.3 %)	
Brenne	0 (0.0 %)	
Mixed	22 (8.5 %)	
Solid	18 (7.0 %)	
Untyped carcinoma	26 (10.1 %)	

Data are given as no. (%) unless otherwise stated

tested for the presence of the most common *BRCA2* mutation (5946delT) using high-resolution melting curve analysis (HRM) [Supplemental Table 2]. Information on HybProbe probe sequences is available upon request. The DNA samples were then genotyped for the nine SNPs in *CTNNB1*, *APC*, and *AXIN2* (Supplemental Table 1 and Supplemental Fig. 1A, Fig. 1B, and Fig. 1C). SNPs were selected with the use of the genome browsers of the International HapMap Consortium (http://www.hapmap.org/index.html.en), University of California Santa Cruz (UCSC; http://genome.ucsc.edu), and dbSNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/). SNPs were selected based on functional significance, location in different LD blocks, and minor allele frequency (MAF) >0.1 in the Caucasian population. Genotyping of the *CTNNB1* rs4533622, rs2953, *APC* rs351771, and *AXIN2* rs4074947, rs3923087, rs2240308 SNPs was performed by HRM on the LightCycler® 480 system (Roche Diagnostics, Mannheim, Germany). Genotyping of the *APC* rs11954856, rs459552, and *AXIN2* rs7224837 SNPs was performed by PCR, followed by the appropriate restriction enzyme digestion [PCR–restriction fragment length polymorphism (PCR-RFLP)] according to the manufacturer's instructions (Fermentas, Vilnius, Lithuania). Primer sequences and conditions for HRM and PCR-RFLP analyses are presented in Supplemental Table 2. Genotyping quality was evaluated by repeated genotyping of 10 % randomly selected samples.

2.3 Statistical Analysis

For each SNP, the Hardy-Weinberg equilibrium (HWE) was assessed by Pearson's goodness-of-fit Chi-square (χ^2) statistic. The differences in the allele and genotype frequencies between cases and controls were determined using standard χ^2 or Fisher tests. The odds ratio (OR) and associated 95 % confidence intervals were also calculated. The data were analyzed under recessive and dominant inheritance models. For the additive inheritance model, SNPs were tested for association with ovarian cancer using the Cochran–Armitage trend test. To adjust for the multiple testing, the Single Nucleotide Polymorphism Spectral Decomposition (SNPpD) method was used [33]. Pair-wise LD between selected SNPs was computed as both D' and r^2 values using HaploView 4.0 software (Broad Institute, Cambridge, MA, USA). Haplotype analysis was performed using the UNPHASED 3.1.5 program with the following analysis options: all window sizes, full model, and uncertain haplotype [34]. Haplotypes with a frequency below 0.01 were set to zero. The p values for both global and individual tests of haplotype distribution between cases and controls were determined. Statistical significance was assessed using the 1,000-fold permutation test.

High-order gene–gene interactions among all tested polymorphic loci were studied by the multifactor dimensionality reduction (MDR) approach (MDR version 2.0 beta 5). A detailed explanation on the MDR method has been described elsewhere [35]. Based on the obtained testing balanced accuracy and cross-validation consistency values, the best statistical gene–gene interaction models were established. A 1,000-fold permutation test was used to assess the statistical significance of MDR models (MDR permutation testing module 0.4.9 alpha).

3 Results

3.1 *BRCA/BRCA2* Mutation Analysis

In patients with ovarian cancer, 30 carriers of the most common *BRCA1* mutations were identified. The *BRCA1* 5382insC mutation was identified in 22 individuals, C61G in five individuals, and 4153delA in three individuals (Supplemental Table 3). None of the patients was a carrier of the *BRCA2* 5946delT mutation. Moreover, none of the controls had the tested *BRCA1/BRCA2* nucleotide variants.

3.2 Association of *CTNNB1*, *APC*, and *AXIN2* Single Nucleotide Polymorphisms with Ovarian Cancer Development in Patients Without *BRCA1/BRCA2* Mutations

The distribution of *CTNNB1*, *APC*, and *AXIN2* genotypes did not display deviation from HWE between patients and control groups ($p > 0.05$). The number of genotypes, ORs, and 95 % confidence interval calculations for the nine *CTNNB1*, *APC*, and *AXIN2* polymorphisms are shown in Table 2.

The lowest p values of the trend test (p_{trend}) were observed for the *APC* rs351771 and rs11954856 SNPs in patients with ovarian cancer ($p_{\text{trend}} = 0.006$ and $p_{\text{trend}} = 0.007$, respectively) [Table 2]. Moreover, we observed that, in a dominant inheritance model, the *APC* rs11954856 SNP is associated with an increased risk of ovarian cancer development [OR = 2.034 (95 % CI 1.302–3.178); $p = 0.002$]. We also found significant allelic differences for the *APC* rs351771 SNP ($p = 0.006$) between patients and controls. The experiment-wide significance threshold required to keep Type I error rate at 5 % was 0.006 (effective number of independent marker loci: 8.388). There was no association of the *APC* rs11954856 SNP (Supplemental Table 4) or other tested SNPs (not shown) with any histologic subtypes of ovarian cancer. Furthermore, none of the other eight *CTNNB1*, *APC*, and *AXIN2* polymorphisms displayed significant association with ovarian cancer development either in dominant, recessive, or additive inheritance models (Table 2).

Table 2 Association of polymorphic variants of *CTNNB1*, *APC*, and *AXIN2* with the risk of ovarian cancer

Gene	rs no.	Alleles[a]	MAF[b]	Genotypes cases[c]	Genotypes controls[c]	$p_{genotypic}$ value	p_{trend} value	$p_{allelic}$ value	$OR_{dominant}$ (95 % CI)[d]; p value	$OR_{recessive}$ (95 % CI)[e]; p value
CTNNB1	rs4533622	a/C	0.46	78/113/37	90/122/70	0.057	0.092	0.082	0.901 (0.622–1.306); 0.583	0.587 (0.376–0.915); 0.018
CTNNB1	rs2953	g/T	0.46	78/113/37	90/122/70	0.057	0.092	0.082	0.901 (0.622–1.306); 0.583	0.587 (0.376–0.915); 0.018
APC	rs11954856	g/T	0.48	35/129/63	76/141/64	0.007	0.007	0.009	**2.034 (1.302–3.178); 0.002**	1.302 (0.871–1.948); 0.198
APC	rs351771	c/T	0.45	88/114/26	86/139/57	0.015	**0.006**	**0.006**	0.698 (0.483–1.009); 0.055	0.508 (0.308–0.839); 0.007
APC	rs459552	a/T	0.30	129/86/13	142/108/32	0.064	0.041	0.034	0.778 (0.548–1.106); 0.161	0.472 (0.242–0.923); 0.025
AXIN2	rs4074947	C/t	0.19	137/80/10	182/89/10	0.577	0.298	0.302	1.208 (0.841–1.734); 0.306	1.249 (0.510–3.056); 0.626
AXIN2	rs7224837	A/g	0.15	161/61/6	203/71/8	0.917	0.801	0.799	1.069 (0.727–1.573); 0.733	0.926 (0.316–2.708); 0.888
AXIN2	rs3923087	a/G	0.22	133/84/10	171/97/14	0.814	0.775	0.777	1.089 (0.763–1.555); 0.640	0.882 (0.384–2.026); 0.767
AXIN2	rs2240308	A/g	0.49	67/115/46	71/146/65	0.510	0.254	0.260	0.809 (0.546–1.197); 0.288	0.844 (0.551–1.292); 0.434

Statistically significant results are highlighted in bold

Experiment-wide significance threshold required to keep Type I error rate at 5 % (Nyholt correction): 0.006 (effective number of independent marker loci: 8.388)

MAF minor allele frequency, *OR* odds ratio, p_{trend} p values of the trend test

[a] Uppercase denotes the more frequent allele in the control samples

[b] Calculated from the control samples

[c] The order of genotypes: DD/Dd/dd (d is the minor allele in the control samples)

[d] Dominant model: dd + Dd vs. DD (d is the minor allele)

[e] Recessive model: dd vs. Dd + DD (d is the minor allele)

3.3 Association of *CTNNB1*, *APC*, and *AXIN2* Haplotypes with Ovarian Cancer Development in Patients Without *BRCA1*/*BRCA2* Mutations

Haplotype analysis of the studied *CTNNB1*, *APC*, and *AXIN2* polymorphisms did not reveal SNP combinations associated with the risk of ovarian cancer development (Table 3). The lowest global $p = 0.025$ was observed for haplotypes composed of the *APC* rs11954856 and rs351771 SNPs (Table 3). However, these results were not statistically significant when permutations were used to generate empiric p values. The empiric 5 % quintile of the best p value after 1,000 permutations was 0.03904 for *CTNNB1*, 0.00748 for *APC*, and 0.00888 for *AXIN2* haplotypes. The *CTNNB1* rs4533622 and rs2953 SNPs were in perfect LD (Supplemental Table 5). The SNPs situated in distinct regions of *APC* and *AXIN2* were either in strong or weak pairwise LD. This was calculated from the control samples, and had D' ranges of 0.401–0.988 for *APC* SNPs and 0.002–1.000 for *AXIN2* SNPs (Supplemental Table 5).

3.4 Multifactor Dimensionality Reduction Analysis of Gene–Gene Interactions Among the Studied *CTNNB1*, *APC*, and *AXIN2* Polymorphisms

Exhaustive MDR analysis evaluating two- to four-loci combinations of all studied SNPs for each comparison did not demonstrate statistical significance in predicting susceptibility to ovarian cancer development (Table 4). The best combination of possibly interactive polymorphisms

Table 3 Results of haplotype analysis of the *CTNNB1*, *APC*, and *AXIN2* genes in patients with ovarian cancer

Polymorphisms	χ^2	Global p value
CTNNB1[a]		
rs4533622_rs2953	3.038	0.386
APC[b]		
rs11954856_rs351771	9.352	0.025
rs351771_rs459552	6.945	0.074
rs11954856_rs351771_rs459552	11.141	0.133
AXIN2[c]		
rs4074947_rs7224837	1.702	0.636
rs7224837_rs3923087	0.245	0.970
rs3923087_rs2240308	2.976	0.395
rs4074947_rs7224837_rs3923087	1.739	0.973
rs7224837_rs3923087_rs2240308	4.037	0.775
rs4074947_rs7224837_rs3923087_rs2240308	7.383	0.946

χ^2 Chi-square

[a] Empirical 5 % quantile of the best p value: 0.03904

[b] Empirical 5 % quantile of the best p value: 0.00748

[c] Empirical 5 % quantile of the best p value: 0.00888

was observed for rs4533622 of *CTNNB1*, rs11954856 of *APC*, and rs7224837 and rs2240308 for *AXIN2* (testing balanced accuracy = 0.5719, cross validation consistency of 10 out of 10, permutation test $p = 0.068$).

4 Discussion

We found that the *APC* rs11954856 polymorphism may be a risk factor of ovarian cancer in Polish population. To date, several polymorphisms in *APC* have been demonstrated to be risk factors of different cancers [24, 25, 30, 31]. The rs454886 SNP (Supplemental Fig. 1B) has been associated with increased breast cancer risk [24]. The *APC* I1307K rs1801155 polymorphism (Supplemental Fig. 1B) has been observed to be associated with an increased risk for colorectal cancer but not breast/ovarian cancers [25, 36, 37]. Moreover, a significant interaction has been found between the D1822V rs459552 (Supplemental Fig. 1B) polymorphism and dietary intakes of high fat, cholesterol, calcium, and fiber for colorectal cancer risk [30, 31].

The β-catenin is the main Wnt pathway effector for which aberrant action has been demonstrated in various cancers [13]. The role of mutations in *CTNNB1* leading to aberrant functioning of β-catenin at the onset of ovarian cancer have been highlighted. There are several studies demonstrating the presence of an oncogenic mutation, mainly located in exon 3 of *CTNNB1*, in upwards of 40 % ovarian endometrioid adenocarcinomas [15–17, 38, 39]. Additionally, several polymorphisms in *CTNNB1* (rs4135385, rs11564475, rs2293303) have been correlated with gastric cancer susceptibility and favorable gastric cancer survival (rs4135385) (Supplemental Fig. 1A) [19]. However, in our studies we did not observe a significant association of the selected *CTNNB1* SNPs with the development of ovarian cancer.

AXIN is a scaffold protein in the destruction complex, enabling the phosphorylation of β-catenin by GSK-3β [40, 41]. Recently, a 64 % allele-specific loss of heterozygosity in *AXIN2* was observed in epithelial ovarian cancer [42]. However, in our studies we did not find an association of the studied *AXIN2* SNPs with ovarian cancer development. Certain polymorphisms located in *AXIN2* have been shown to be risk factors of astrocytoma (rs1133683), lung cancer (rs2240308), and prostate cancer (rs35285779) [Supplemental Fig. 1C] [26–29]. In addition to these findings, the five *AXIN2* SNPs rs7210356, rs4791171, rs11079571, rs3923087, and rs3923086 were associated with an increased risk of breast cancer (Supplemental Fig. 1C) [24].

Table 4 Results of gene–gene interactions analyzed by multifactor dimensionality reduction method

Polymorphisms	Testing balanced accuracy	Cross validation consistency (%)	p value[a]
*CTNNB1*_rs4533622, *APC*_rs11954856	0.5418	60	0.356
*CTNNB1*_rs4533622, *APC*_rs351771, *AXIN2*_rs4074947	0.5041	50	0.828
*CTNNB1*_rs4533622, *APC*_rs11954856, *AXIN2*_rs7224837, *AXIN2*_rs2240308	0.5719	100	0.068

[a] Significance of accuracy (empirical p value based on 1,000 permutations)

5 Conclusion

Our study demonstrated significantly increased *APC* rs11954856 and rs351771 SNPs frequencies in Polish women with ovarian cancer. These SNPs were not identified in recently conducted genome-wide association studies (GWAS) as risk factors for ovarian cancer, probably due to genome-wide significance thresholds required for a conventional GWAS (p value $<5 \times 10^{-8}$) [43–45]. The association of intronic rs11954856 and synonymous rs351771 (Ala545Ala) SNPs with ovarian cancer may be due to LD with one or more functional polymorphisms of *APC*. The lack of association of *CTNNB1* and *AXIN2* SNPs

with ovarian cancer might be due to the small sample size. Therefore, to confirm the role of the studied SNPs in ovarian cancer, this study should be replicated in a larger and independent cohort, and functional studies of these SNPs must be performed.

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Conflict of Interest The authors declare that they have no conflict of interest.

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