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The *MDM2* Promoter SNP285C/309G Haplotype Diminishes Sp1 Transcription Factor Binding and Reduces Risk for Breast and Ovarian Cancer in Caucasians

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SUMMARY

MDM2 plays a key role in modulating p53 function. The *MDM2* SNP309T > G promoter polymorphism enhances Sp1 binding and has been linked to cancer risk and young age at diagnosis although with conflicting evidence. We report a second *MDM2* promoter polymorphism, SNP285G > C, residing on the SNP309G allele. SNP285C occurs in Caucasians only, where 7.7% (95% CI 7.6%–7.8%) of healthy individuals carry the SNP285C/309G haplotype. In vitro analyses reveals that SNP309G enhances but SNP285C strongly reduces Sp1 promoter binding. Comparing *MDM2* promoter status among different cohorts of ovarian (n = 1993) and breast (n = 1973) cancer patients versus healthy controls (n = 3646), SNP285C reduced the risk of both ovarian (OR 0.74; CI 0.58–0.94) and breast cancer (OR 0.79; CI 0.62–1.00) among SNP309G carriers.

Significance

The *MDM2* promoter polymorphism SNP285C diminishes Sp1 transcription factor binding and reduces risk of breast and ovarian cancer among Caucasians. SNP285C, which forms a distinct haplotype with SNP309G, is a young polymorphism that seems to rapidly expand within the Caucasian population; in our study, 7.7% out of 3991 healthy individuals carried the SNP285C/309G haplotype. The distribution of the SNP285C/309G haplotype between populations provides information considering phylogenetic selection of *MDM2* variants. Further, SNP285C may explain why cancer risk studies related to *MDM2* SNP309 status have provided different results in Caucasian compared with Asian populations.

Table 1. Distribution of *MDM2* Promoter Haplotypes (Alleles) with Respect to SNP285 and SNP309 in Healthy Controls from Different Populations

Haplotype	Norway[a]		Netherlands[a]		UK		Finland		China	
285–309	*n*	(%)	*n*	(%)	*n*	(%)	*n*	(%)	*n*	(%)
G-T	1739	(65.0)	1565	(66.3)	452	(65.5)	205	(56.3)	347	(54.4)
G-G	826	(30.9)	704	(29.8)	213	(30.9)	156	(42.9)	291	(45.6)
C-G	109	(4.1)	93	(3.9)	25	(3.6)	3	(0.8)	0	(0)
C-T	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)
Total	2674	(100)	2362	(100)	690	(100)	364	(100)	638	(100)

See also Tables S1–S3.
[a] Identical to cohorts used for cancer risk evaluation (Tables 3 and 4).

INTRODUCTION

The MDM2 (Mouse Double Minute 2 homolog) phosphoprotein plays a central function in cell cycle control. It binds to and inhibits the function of the pivotal growth arrest and apoptosis regulator p53 (the protein coded for by the *TP53* gene) and interacts with several other major proteins involved in these cellular processes, like pRb (Xiao et al., 1995) and E2F1 (Martin et al., 1995). *MDM2* knockout leads to embryonic death in mice, a characteristic that is diminished in *MDM2*/*TP53* double-knockouts (Toledo and Wahl, 2006).

MDM2 amplification has been considered as an alternative mechanism of p53 inactivation in several tumor forms (Momand et al., 1992; Oliner et al., 1992). Subsequently, a polymorphism (SNP309T > G; rs2279744) in the *MDM2* intronic promoter (P2) was found to enhance Sp1 transcription factor binding and to associate with increased MDM2 expression (Bond et al., 2004). The 309G polymorphism has been related to early cancer development among individuals harboring *TP53* germline mutations (Li-Fraumeni syndrome), young age at diagnosis of soft tissue sarcomas, as well as "estrogen receptor (ER) rich" breast cancer among women with wild-type *TP53* (Bond et al., 2004, 2006). A recent study revealing $MDM2^{SNP309G/G}$ mice to be more tumor prone than $MDM2^{SNP309T/T}$ mice (Post et al., 2010) further corroborated the impact of the SNP309G allele on cancer risk. However, case-control studies linking this polymorphism to risk of tumor development and age of diagnosis in different types of solid tumors have provided conflicting results, with a bias toward positive associations in Asian populations and negative results among Caucasians (Economopoulos and Sergentanis, 2010; Hu et al., 2007), including a large study of >5000 Western European breast cancer patients (Schmidt et al., 2007). So far, studies of ovarian cancer have been small (Campbell et al., 2006; Galic et al., 2007; Kang et al., 2009; Ueda et al., 2009) and some have been restricted to *BRCA1/2* mutation carriers (Copson et al., 2006; Nechushtan et al., 2009; Yarden et al., 2008).

Following our discovery of a second *MDM2* promoter polymorphism, SNP285, we aimed at exploring this variant's effect on Sp1 binding, its distribution between different ethnic groups and its impact on risk of breast and ovarian cancer.

RESULTS

MDM2 Promoter Polymorphisms

Studying the *MDM2* 309T > G promoter polymorphism in Norwegian cancer patients and healthy controls we observed four nucleotide substitutions in the intronic promoter of *MDM2* (promoter P2). In addition to the previously reported SNP309T > G, we observed two individuals harboring an adenine in this position (one healthy control harboring a SNP309TA genotype and one ovarian cancer patient harboring a SNP309GA genotype). We also observed one healthy control harboring a G to A transition 14 bp upstream of SNP309 (position 295). Due to the fact that these two variants were observed in only two and one out of several thousand individuals, no further studies were performed with emphasis on these rare variants.

Concomitantly with a Scottish group (Paulin et al., 2008), we discovered a G to C transversion located only 24 bp upstream of SNP309 (SNP285) in the *MDM2* intronic promoter (P2). Based on initial screenings, we estimated the frequency of this variant among Norwegian individuals to be approximately 8%. SNP285C was observed in individuals harboring the SNP309G only (Table 1), and subsequent statistical calculations based on the distribution of SNP285C across large Norwegian and Dutch cohorts strongly indicate SNP285C/309G to be a distinct haplotype ($p < 1.0 \times 10^{-10}$).

SNP285G>C Diminishes Sp1 Transcription Factor Binding to the *MDM2* Promoter

To assess potential biological effects of the SNP285C, we evaluated Sp1 binding by in silico predictions using the JASPAR database (Sandelin et al., 2004). While this analysis predicted SNP309G to enhance Sp1 binding by extending a Sp1 binding site (as previously reported by Bond et al., 2004), interestingly, SNP285C was predicted to reduce Sp1 binding at an adjacent binding site.

Next, we determined the binding strength between Sp1 and the different *MDM2* promoter haplotypes in vitro, through assays measuring changes in surface plasmon resonance (SPR). In these binding analyses, double-stranded DNA 104 mers representing the different variants of the *MDM2* promoter were injected into a flow over the ligand Sp1 protein linked to a chip (Figure 1). DNA binding to the Sp1-coated chip, as opposed to reference, confirmed recombinant Sp1 to retain its DNA binding capability after being linked to the chip surface.

Notably, Sp1-*MDM2*-promoter binding curves did not fit a 1:1 binding model; the curves were found best fitted to a heterogeneous ligand model, indicating more than one Sp1 molecules to bind each *MDM2*-promoter fragment. This finding is consistent with the in silico analysis predicting SNP285 and SNP309 to be located in two adjacent Sp1 binding sites.

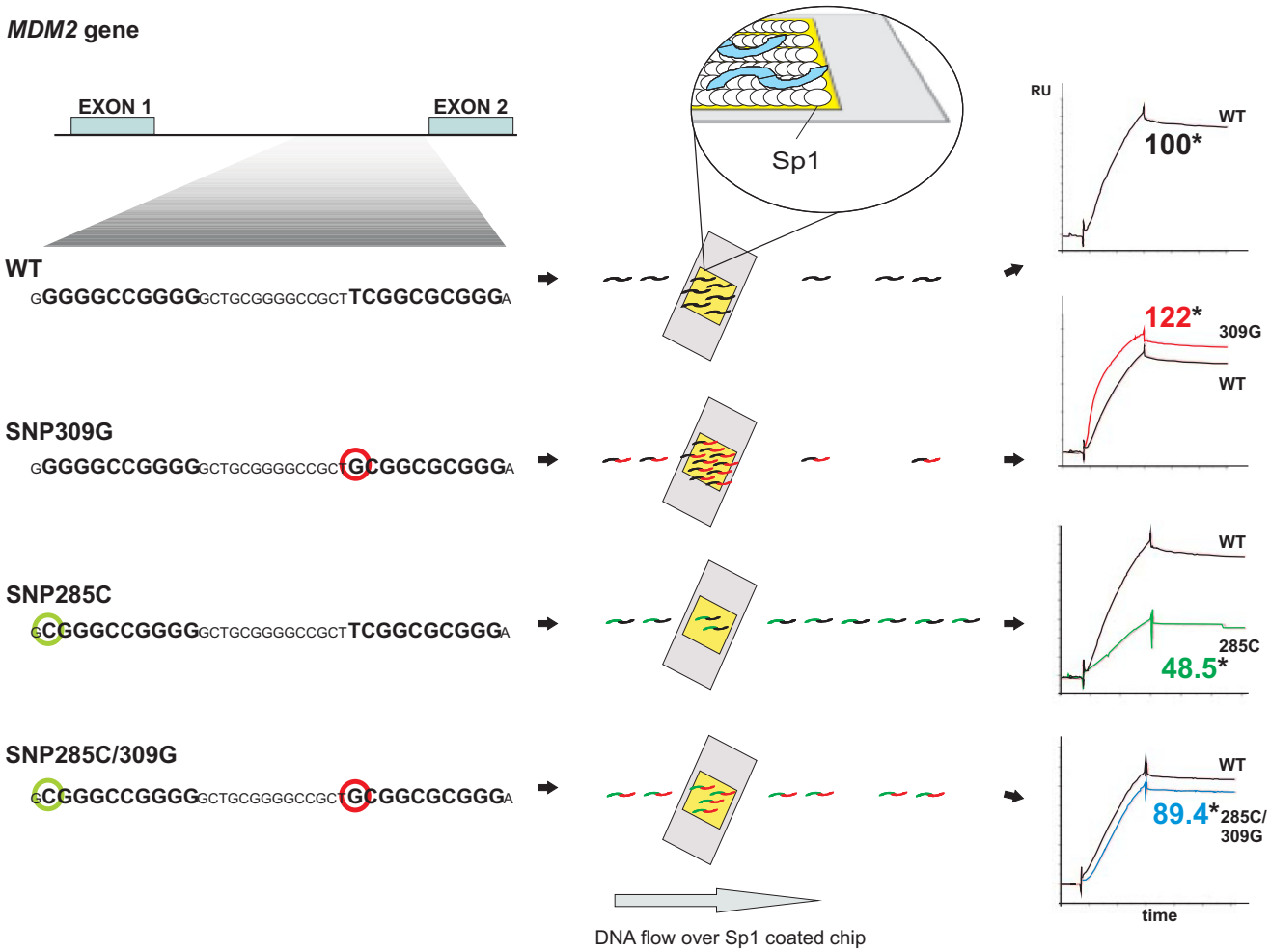


Figure 1. In Vitro Assay for the Effect of SNP285 and SNP309 on Sp1 Binding to the *MDM2* Promoter

DNA fragments containing a small element of the intronic *MDM2* promoter, P2 (position 284–319 downstream of exon 1, linked to phage-lambda DNA, increasing fragment size to 104 bp) were amplified by PCR. Predicted Sp1 binding sites within this element are denoted in bold. Fragments containing SNP285C (encircled green) and SNP309G (encircled red) were used together with recombinant human Sp1 protein in biomolecular interaction analyses (BIA), performed on the BIACORE T100. In brief, the DNA fragments were passed over a Series S sensor chip CM5 coated with Sp1. DNA binding to Sp1 causes changes in the surface plasmon resonance (SPR), which was recorded as RUs (1 RU corresponds to 10^{-6} refractive index units). Based on SPR, binding curves were generated and calculations of relative binding strengths were performed using the BIA evaluation software version 2.0.1. *Numbers indicate relative binding strength, as percentage of wild-type (WT).

Using the "wild-type" SNP285G/309T haplotype as reference (100%), pairwise analysis revealed SNP309G to enhance Sp1 protein binding to 122%, confirming previous results obtained with a different technique (Bond et al., 2004). In contrast, SNP285C reduced Sp1 binding to only 48.5% of that of the SNP285G/309T haplotype. Importantly, a fragment representing the double polymorphic SNP285C/309G haplotype bound Sp1 with an affinity of 89.4% of that of the wild-type SNP285G/309T haplotype (Figure 1). All differences in relative binding strength were confirmed by replicates and separate pairwise analyses of all possible combinations of the polymorphic variants on the same chip (12 different experiments in total).

SNP285G>C Is Present in Caucasian Populations Only

To evaluate the global distribution of SNP285C, we screened for this polymorphism in an extended group of Norwegian healthy individuals (n = 1337), closely related Caucasian populations (Dutch; n = 1181 and British; n = 345), a Caucasian population with a different historical migratory pattern (Finnish; n = 182), and an Asian group (Chinese; n = 319). These data are summarized in Tables 1 and 2. A detailed overview of SNP285 genotypes within the different SNP309 genotype-groups is given in Table S1 (available online) and subcohorts of Norwegian and Dutch healthy controls are described in Tables S2 and S3.

The SNP285C/309G haplotype accounted for 11.7% of the SNP309G alleles both among Norwegians and Dutch individuals, 10.5% among British, and 1.9% among Finnish individuals. Accordingly, 7.8% of the Norwegian, 7.8% of the Dutch and 7.2% of the British individuals carried this allele, in contrast to 1.6% in the Finnish population. Thus, the prevalence of SNP285 in Norway, The Netherlands and UK combined was 7.7% (95% CI 7.6%–7.8%) and no difference in the frequency was observed

Table 2. Distribution of *MDM2* SNP285 Genotypes among Healthy Individuals from Different Populations

	Norway[a]		Netherlands[a]		UK		Finland		China	
SNP285 Genotype	*n*	(%)	*n*	(%)	*n*	(%)	*n*	(%)	*n*	(%)
GG	1233	(92.2)	1089	(92.2)	320	(92.8)	179	(98.4)	319	(100)
GC	99	(7.4)	91	(7.7)	25	(7.2)	3	(1.6)	0	(0)
CC	5	(0.4)	1	(0.1)	0	(0)	0	(0)	0	(0)
Total	1337	(100)	1181	(100)	345	(100)	182	(100)	319	(100)

See also Tables S1–S3.
[a] Identical to cohorts used for cancer risk evaluation (Tables 3 and 4).

between these countries (p = 0.871). In contrast, the frequency in Finland was significantly lower as compared with the three former countries (p = 0.002; Chi-square; multiple comparison). Importantly, SNP285C was not observed among Chinese individuals.

Potential Effects of SNP309 and SNP285 on Cancer Risk

Based on our in vitro results, we hypothesized that SNP309G may be associated with a moderately elevated cancer risk. In contrast, the profound effect of SNP285C on Sp1 binding suggests this polymorphism to reduce cancer risk. Assuming SNP285C to antagonize the effect of SNP309G, this may explain contemporary findings indicating SNP309G to increase cancer risk in most ethnic cohorts but not among Caucasians (Economopoulos and Sergentanis, 2010; Hu et al., 2007). To assess a potential effect of SNP285C on cancer risk, we generated two test hypotheses. First, we postulated that SNP285C diminished the effect of SNP309G on cancer risk among Caucasians. This hypothesis could be tested by comparing the effect of SNP309G status on cancer risk in repeated analysis excluding individuals harboring the SNP285C/309G haplotype. Second, we postulated that the SNP285C/309G haplotype reduced cancer risk among SNP309TG heterozygotes and/or 309GG homozygotes. Thus, we tested these hypotheses in large cohorts of patients diagnosed with either breast or ovarian cancer.

SNP285G>C Reduces Risk of Breast Cancer

We genotyped *MDM2* SNPs in two unselected breast cancer cohorts; one Norwegian (n = 956) and one Dutch (n = 1017). The SNP distributions were compared with representative healthy control samples obtained from the same countries (Norway; n = 1337, The Netherlands; n = 1181). Breast cancer patients were unselected for family history of their disease.

The frequencies of *MDM2* promoter haplotypes were significantly different among breast cancer patients and healthy controls (p = 0.021) (Table 3). Consistent with the findings of others in Caucasian populations (Schmidt et al., 2007), we found no increased breast cancer risk associated with harboring the SNP309G allele (Table 4). However, excluding individuals carrying the SNP285C, we found the risk of breast cancer to be significantly increased among SNP309G carriers (Mantel-Haenszel (M-H) test adjusted for countries OR 1.15; CI 1.01–1.30). This finding is consistent with our hypothesis suggesting the contribution of SNP285C to neutralize the effect of SNP309G on cancer risk among Caucasians. Notably, the association was higher among SNP309GG homozygotes (OR 1.27; CI 1.03–1.55) as compared with SNP309TG heterozygotes (OR 1.11; CI 0.98–1.27).

SNP285C reduced risk of breast cancer among SNP309G allele carriers (SNP309TG heterozygotes and SNP309GG homozygotes combined; M-H test adjusted for countries; OR 0.79; CI 0.62–1.00). Interestingly, subgroup analysis revealed the effect of SNP285C to be significant among SNP309GG homozygotes (M-H; OR 0.55; CI 0.35–0.86) with a minor, non-significant trend among SNP309TG heterozygotes (M-H; OR 0.91; CI 0.69–1.20) (Figure 2). Testing for heterogeneity between Dutch and Norwegian breast cancer patients revealed no significant difference with respect to SNP285C distribution (p = 0.521), and the data combined revealed a significantly lower SNP285C/309G haplotype frequency among breast cancer patients (9.3%) as compared with healthy controls (11.7%; p = 0.029).

SNP285G>C Reduces Risk of Ovarian Cancer

Initially, we genotyped 832 Norwegian patients diagnosed with an epithelial ovarian carcinoma. Ovarian cancer patients were

Table 3. Distribution of *MDM2* Promoter Haplotypes (with respect to SNP285 and SNP309) in Ovarian and Breast Cancer Cases versus Healthy Controls

Haplotype	Ovarian Cancer		Breast Cancer		Healthy[a]	
285–309	*n*	(%)	*n*	(%)	*n*	(%)
G-T	1029	(61.8)	2520	(63.9)	3304	(65.6)
G-G	584	(35.1)	1294	(32.8)	1530	(30.4)
C-G	51	(3.1)	132	(3.3)	202	(4.0)
C-T	0	(0)	0	(0)	0	(0)
Total	1664	(100)	3946	(100)	5036	(100)
	p = 0.001[b]		p = 0.021[b]			

[a] Total of Norwegian and Dutch cohorts presented in Tables 1 and 2.
[b] Compared with healthy controls (Chi square).

Table 4. Effect of *MDM2* SNP309 on Breast and Ovarian Cancer

SNP309 Genotype	SNP285 Genotype	Breast Cancer		Ovarian Cancer		Healthy[a]
		n	OR (95% CI)[b]	*n*	OR (95% CI)[c]	*n*
TT	GG	805	1.00	296	1.00	1090
TG	GG	813	1.11 (0.98–1.27)	406	1.42 (1.17–1.71)	993
TG	GC	97	1.01 (0.77–1.34)	31	0.81 (0.52–1.25)	131
TG	GG+GC[d]	910	1.10 (0.97–1.25)	437	1.34 (1.11–1.62)	1124
GG	GG	225	1.27 (1.03–1.55)	80	1.19 (0.87–1.62)	239
GG	GC	33	0.69 (0.45–1.06)	19	1.16 (0.65–2.09)	65
GG	GG+GC[d]	258	1.14 (0.95–1.38)	99	1.18 (0.89–1.57)	304
TG+GG[d]	GG	1038	1.15 (1.01–1.30)	486	1.37 (1.14–1.65)	1232
TG+GG[d]	GC	130	0.90 (0.71–1.15)	50	0.91 (0.63–1.31)	196
TG+GG[d]	GG+GC[d]	1168	1.11 (0.99–1.25)	536	1.31 (1.10–1.57)	1428

[a] Total of Norwegian and Dutch cohorts presented in Tables 1 and 2.
[b] OR compared with healthy controls, calculated as common odds ratio adjusted for countries.
[c] OR compared with initial cohort of Norwegian healthy controls (presented in Tables 1 and 2). For further details on SNP distribution in this cohort and the validation set of Norwegian healthy controls, see Table S1.
[d] Combined groups.

unselected for family history, but individuals with mutations related to hereditary ovarian cancer or Lynch syndromes were not included.

Similar to what was observed among breast cancer patients, the distribution of *MDM2* promoter haplotypes was significantly different among ovarian cancer patients compared with healthy controls (p = 0.001) (Table 3).

We observed an increased risk of ovarian cancer among SNP309G carriers, in particular among individuals with the SNP309TG genotype (Table 4). Removing carriers of the SNP285C/309G haplotype from the analyses, the association between SNP309G and risk for ovarian cancer was strengthened, consistent with our hypothesis suggesting SNP285C to act as an antagonist toward SNP309G (Table 4).

As for breast cancer, SNP285C significantly reduced the risk of ovarian cancer among SNP309G allele carriers (SNP309GG homozygotes and SNP309TG heterozygotes combined: OR 0.67; CI 0.47–0.95) (Figure 2). Stratifying for genotype, a strong

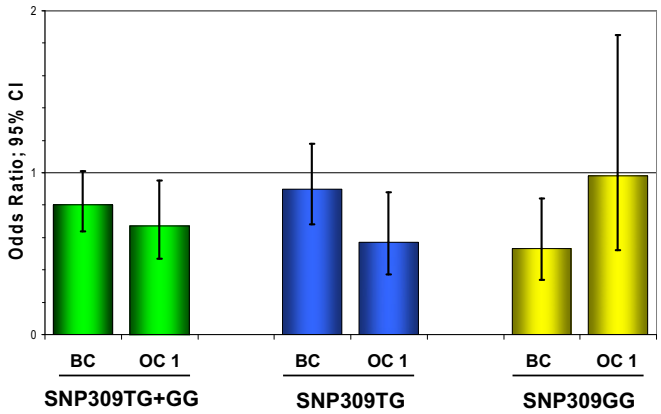
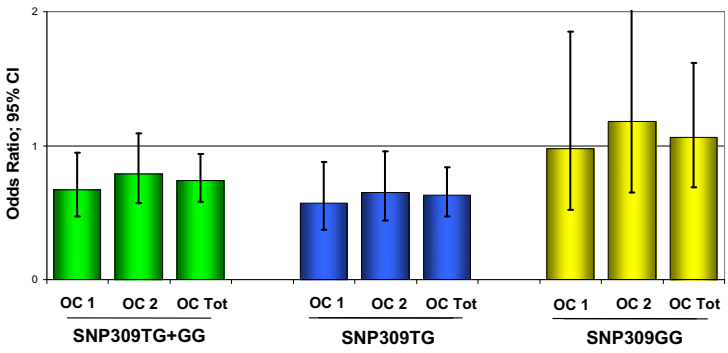


Figure 2. SNP285C Impact on Risk for Breast and Ovarian Cancer

Effect of SNP285C on risk for breast (BC) and ovarian (OC) cancer, versus healthy controls (HC), among individuals with SNP309TG and/or GG genotype. Error bars indicate 95% CI. [1,2,3]Among these individuals 6(1), 2(2), and 1(3) harbored the SNP285CC genotype. [4]Calculated as common odd ratio by Mantel-Haenszel test, adjusting for potential differences between Norwegian and Dutch cohorts. [5]Fischer exact test calculated based on allelic frequencies.

	HC	BC	OC 1	HC	BC	OC 1	HC	BC	OC 1
SNP285GG									
n	1232	1038	486	993	813	406	239	225	80
(%)	**(86.3)**	**(88.9)**	**(90.7)**	**(88.3)**	**(89.3)**	**(92.9)**	**(78.6)**	**(87.2)**	**(80.8)**
SNP285GC									
n	196	130	50	131	97	31	65	33	19
(%)	**(13.7)[1]**	**(11.1)[2]**	**(9.3)[3]**	**(11.7)**	**(10.7)**	**(7.1)**	**(21.4)[1]**	**(12.8)[2]**	**(19.2)[3]**
OR		0.79[4]	0.67		0.91[4]	0.57		0.55[4]	0.98
CI (95%)		0.62 1.00	0.47 0.95		0.69 1.20	0.37 0.88		0.35 0.86	0.52 1.85
P-value[5]		0.031	0.011		0.525	0.007		0.006	0.606



	HC 2	OC 1	OC 2	OC Tot	HC 2	OC 1	OC 2	OC Tot	HC 2	OC 1	OC 2	OC Tot
SNP285GG												
n	530	486	621	1107	412	406	486	892	118	80	135	215
(%)	**(85.9)**	**(90.7)**	**(88.6)**	**(89.5)**	**(86.6)**	**(92.9)**	**(90.8)**	**(91.8)**	**(83.7)**	**(80.8)**	**(81.3)**	**(81.9)**
SNP285GC												
n	87	50	80	130	64	31	49	80	23	19	31	50
(%)	**(14.1)**[1]	**(9.3)**[2]	**(11.4)**[3]	**(10.5)**[4]	**(13.4)**	**(7.1)**	**(9.2)**	**(8.2)**	**(16.3)**[1]	**(19.2)**[2]	**(18.7)**[3]	**(18.9)**[4]
OR		0.67[5]	0.79[6]	0.74[7]		0.57[5]	0.65[6]	0.63[7]		0.98[5]	1.18[6]	1.06[7]
CI (95%)		0.47 0.95	0.57 1.09	0.58 0.94		0.37 0.88	0.44 0.96	0.47 0.84		0.52 1.85	0.65 2.13	0.69 1.62
P-value[8]		0.011	0.172	0.011		0.007	0.036	0.002		0.606	0.591	1.000

Figure 3. SNP285C Impact on Risk of Ovarian Cancer in Independent Data Sets

Bars represent the OR calculated for the initial cohort of ovarian cancer patients (OC 1) versus healthy controls (HC 1), the independent cohort of ovarian cancer patients (OC2) versus healthy controls (HC 2) in addition to the corresponding values for pooled cohorts (OC Tot) among individuals with SNP309TG and/or GG genotype. Error bars indicate 95% CI.[1,2,3,4] Among these individuals, 3(1), 1(2), 4(3), and 5(4) harbored the SNP285CC genotype.[5] OR calculated from initial cohort of ovarian cancer patients (OC 1) versus initial cohort of healthy controls; as presented in Figure 2. [6]OR calculated from verification set of ovarian cancer patients (OC 2) versus verification set of healthy controls (HC 2). [7]OR calculated from total of all ovarian cancer patients, including the initial cohort presented in Figure 2. (OC Tot; = OC 1 + OC 2) versus total of Norwegian healthy controls, including the initial cohort presented in Figure 2. [8]Fischer exact test calculated based on allelic frequencies.

effect was observed among individuals carrying the SNP309TG genotype (OR 0.57; CI 0.37–0.88) while no significant association was observed in the SNP309GG group (OR 0.98; CI 0.52–1.85). However, only 19 ovarian cancer patients were carriers of the SNP285GC/309GG genotype, and the result should be interpreted carefully with respect to this subgroup.

The frequency of the SNP285C/309G haplotype among SNP309G alleles was significantly lower among Norwegian ovarian cancer patients (8.0%) as compared with healthy Norwegians (11.7%; p = 0.022) and Dutch (11.7%; p = 0.027) controls (combining Norwegian and Dutch control groups; p = 0.011).

SNP285G>C Reduces Risk of Ovarian Cancer in Independent Patient and Control Cohorts

In order to verify the effect of SNP285C on ovarian cancer risk, we genotyped SNP285 and SNP309 across an independent cohort of Norwegian ovarian cancer patients and a second cohort of healthy Norwegian controls. Here, we wanted to confirm the hypothesis that SNP285C is associated with reduced risk of ovarian cancer among carriers of the SNP309TG genotype. To estimate the sample size needed for this validation, we took advantage of the results obtained in our initial cohorts where SNP285C was observed in 7.1% of ovarian cancer patients and in 11.8% of healthy controls carrying the SNP309TG genotype. Using a one-sided alpha-value of 0.05 and a beta-value of 0.8, the required number of ovarian cancer patients and healthy controls, harboring the SNP309TG genotype, was 512 in each group. To obtain this number with a probability >90%, a total number of 1018 ovarian cancer patients and 1153 healthy controls were needed (for full details on calculations, see Supplemental Experimental Procedures). Obtaining and analyzing DNA from 1161 new ovarian cancer patients and 1128 new healthy controls, these criteria were fulfilled.

While the frequency of SNP285C in the control validation cohort (7.7%) mirrored the frequency in our first Norwegian healthy control cohort (7.8%), the prevalence of SNP285C among ovarian cancer patients in the validation set (6.9%) was slightly higher than in our initial cohort (6.0%), but the difference was not statistically significant (p = 0.46).

Analyzing the validation sets of ovarian cancer patients and healthy controls, SNP285C significantly reduced the risk of ovarian cancer among SNP309TG heterozygous individuals (OR 0.65; CI 0.44–0.96). In contrast, SNP285C had no effect on ovarian cancer risk among SNP309GG homozygous individuals (OR 1.18; CI 0.65–2.13) (Figure 3). Combining the data from our initial cohorts and the validation sets, SNP285C strongly reduced the risk of ovarian cancer among individuals harboring the SNP309TG genotype (OR 0.63; CI 0.47–0.84).

The differential effect of SNP285C on SNP309TG heterozygotes versus SNP309GG homozygotes with respect to ovarian cancer risk mirrors the effect observed in our initial dataset (Figure 3). This contrasts with the results from the breast cancer cohort, where the effect of SNP285C was observed among individuals carrying the SNP309GG but not the SNP309TG genotype (Figure 2). Importantly, patients in the validation cohort harboring the SNP285GG/309TG genotype revealed an elevated risk of ovarian cancer as compared with individuals harboring the SNP285GG/309TT genotype (OR 1.31; CI 1.09–1.57), again consistent with the results from our first dataset. A differential effect of *MDM2* status in ovarian and breast cancer was further corroborated by the finding of SNP285C disequilibrium comparing breast and ovarian cancer patients (initial ovarian cancer cohort: p = 0.027; verification cohort: p = 0.061; calculations based on data presented in Figures 2 and 3).

Additionally, we analyzed a small cohort of British ovarian cancer patients (n = 115). Including these patients (with 345

British controls) in a Mantel-Haenszel (M-H) test adjusted for country did not substantially influence the association (OR 0.67; CI 0.51–0.89 among SNP309TG heterozygotes).

Potential Correlations between *MDM2* SNP Status, p53 Arg72Pro, ER Status, and Age at Breast Cancer Diagnosis

Some studies (Ashton et al., 2009; Ellis et al., 2008; Nunobiki et al., 2009) have reported a potential interaction between the p53 Arg72Pro polymorphisms and the *MDM2* SNP309G allele. Analyzing *TP53* status among 312 breast cancer patients in this study, we found no correlation between either of the two *MDM2* promoter polymorphisms and the *TP53* Arg72Pro status ($p > 0.40$ for all comparisons).

Interestingly, Bond et al. (2006) detected a correlation between age at diagnosis in breast cancer patients harboring estrogen receptor (ER)-rich breast tumors, defined as having >50% of the tumor cells staining positively for the ER. Our data did not allow a similar subgroup analysis. However, relating age at diagnosis to *MDM2* SNP309 and SNP285 status among 330 breast cancer patients defined as ER positive by conventional criteria (>10% of tumor cells staining positive), we detected no correlation between *MDM2* SNP status and age at breast cancer diagnosis ($p > 0.40$ for all comparisons).

DISCUSSION

Germline mutations in high-penetrance genes including *BRCA1*, *BRCA2*, and (rarely) other genes like *TP53* are associated with a high risk of breast cancer. Similarly, *BRCA1* and *BRCA2* mutations are associated with high risk of ovarian cancer. However, confirmed germline mutations account for less than 5% of all breast cancers and about 10% of ovarian cancers (for references, see ACOG 2009). Thus, recent research has focused on polymorphisms in "low-risk" genes and their combinations to explain inherited cancer predisposition. While genome-wide analysis studies (GWAS) have identified polymorphisms in different genes to be associated with elevated breast and ovarian cancer risk, except for a few genes including *FGFR2* and *TNRC9* (Easton et al., 2007; Yang et al., 2009) conferring enhanced breast cancer risk of 35% and 28%, such polymorphisms are associated with a minor risk increase in the range of 10%–15% (for references, see Yang et al., 2009).

Importantly, for most genes related to breast and ovarian cancer risk through GWAS, the functional explanation to their effects is, at best, incomplete. This contrasts the well-established knowledge about the mechanisms by which MDM2 executes its effects on key genes involved in apoptosis as well as growth arrest (Toledo and Wahl, 2006). The importance of *MDM2* status with respect to cancer growth is illustrated by the frequent findings of *MDM2* overexpression, caused by gene amplifications or other mechanisms in several tumor forms (for references, see Bartel et al., 2001). A milestone in this research area was reached with the discovery by Arnold Levine's group in 2004 of the *MDM2* promoter polymorphism SNP309T > G enhancing *MDM2* expression by increasing Sp1 promoter binding (Bond et al., 2004) and subsequent studies corroborating the role of SNP309G as a cancer risk modulator (Bond et al., 2006; Post et al., 2010). While Bond et al. (2004) reported the effects of the human *MDM2* promoter variants in *Drosophila* cell lines, this study addresses transcription factor binding to the *MDM2* promoter by use of SPR. Our results revealed SNP309G to enhance Sp1 binding, while SNP285C and the combined SNP285C/309G haplotype both reduced Sp1 binding. The strong effect of the SNP285C polymorphism as compared with SNP309G on Sp1 binding strength is corroborated by the different impact of the two polymorphisms on cancer risk. While additional *MDM2* promoter polymorphisms located on the 309T allele have been reported in different ethnic groups (Atwal et al., 2007), the functional effects of these variants remain unknown.

Characterization of the 285C polymorphism with respect to molecular effects, ethnic distribution, and impact on ovarian and breast cancer risk expands our understanding of the role of *MDM2* allele variants substantially. We propose that occurrence of the 285C polymorphism among Western Caucasians may account for the discrepancy in previous studies evaluating the effects of SNP309G in different ethnic populations (Economopoulos and Sergentanis, 2010; Hu et al., 2007) on cancer risk. The finding underlines the need of careful interpretations of disease odds ratios as well as genome-wide associations obtained from pooled populations with a different ethnic background, or located in different geographical areas.

Secondary somatic mutations counteracting the effect of a primary mutation has recently been described as a mechanism circumventing drug sensitivity in cancer cells (Edwards et al., 2008; Sakai et al., 2008). The present observation of a second polymorphism antagonizing the effects of another polymorphism located on the same allele, illustrates similar mechanisms in a much longer time frame.

Importantly, we observed a significantly reduced risk of ovarian, but not breast cancer related to SNP285C among SNP309TG heterozygotes. Consistent with this finding, we observed an increased risk of ovarian, but not breast cancer among individuals harboring the SNP285GG/309TG genotype (Figure 1 and Table 4). Importantly, the significantly different effect of SNP285C on SNP309TG heterozygotes and SNP309GG homozygotes with respect to ovarian cancer risk was detected in two large independent data sets. Taken together, these data support the hypothesis that heterozygosity for SNP309G, and the neutralizing effect of SNP285C, have significant impact on ovarian cancer risk. In contrast, the effect of SNP285C on breast cancer risk was restricted mainly to individuals carrying the SNP309GG genotype. A differential effect of *MDM2* status in ovarian and breast cancer was corroborated by the finding of SNP285C disequilibrium ($p = 0.027$) comparing breast and ovarian cancer patients (Figure 2). While it is well known that heterozygous carriers of recessive genes may be at risk of diseases different from the major disease observed in homozygotes (Smirnov and Cheung, 2008), we here show that such disparity also may be found with respect to risk of different cancer forms.

Our findings of a second *MDM2* promoter polymorphism (SNP285C) antagonizing the effect of a MDM2 enhancing polymorphism (SNP309G) may lead to speculations on evolutionary mechanisms selecting for different *MDM2* alleles and the role of the MDM2 – p53 axis in human biology beyond cancer risk. SNP285C seems to be a young polymorphism that has arisen in Western Europe. With the very low frequency of SNP285C observed among Finnish individuals, most likely this

polymorphism has originated after the major migration toward Finland and Western European countries including the United Kingdom, Holland, and Norway (Peltonen et al., 1999), This contrasts the ancient 309G polymorphism, found in all ethnic groups. The fact that SNP285C has already spread to 7.7% of the Western Caucasian individuals indicate positive selection for this polymorphism through influence on factors related to survival and/or reproductive biology. Notably, SNP309G has been linked to risk of missed abortion (Fang et al., 2009). Whatever the mechanisms may be, the fact that age at cancer diagnosis in general exceeds reproductive age suggests mechanisms other than cancer risk to be important for the selection of *MDM2* variants.

Regulation of p53 function involves several genes like *MDM2*, *ATM*, *CHEK2*, and the Nijmegen Breakthrough Syndrome Gene (NBS1). The fact that alterations of these genes may partly substitute for *TP53* mutations with respect to modulation of cancer risk, tumor progression and response to cytotoxic stress (Buscemi et al., 2001, 2004; Chrisanthar et al., 2008; Meyn, 1999; Oliner et al., 1992; Varon et al., 2001) underlines the critical importance of tight control of the entire p53 functional pathway. While *TP53* itself harbors a frequent polymorphism (Arg72Pro), evidence linking this variant to increased cancer risk is conflicting (Francisco et al., 2010). Some studies have linked *MDM2* SNP309 to the *TP53* polymorphism Arg72Pro (Ashton et al., 2009; Ellis et al., 2008; Nunobiki et al., 2009). However, we found no association between either of the two *MDM2* polymorphisms and *TP53* Arg72Pro status in our data. This observation is in line with the results from the Breast Cancer Association Consortium (Schmidt et al., 2007).

In conclusion, *MDM2* SNP285C significantly reduced the risk of breast and ovarian cancer among Caucasians, and revealed ethnic genetic diversity with respect to the risk of these two malignancies. The finding of a late polymorphism (SNP285C) antagonizing an ancient polymorphism (SNP309G) draws attention to the mechanisms that have driven the phylogenetic selection of *MDM2* variants.

EXPERIMENTAL PROCEDURES

In Silico Predictions of Sp1 Binding to the *MDM2* Promoter

Prediction of Sp1 transcription factor binding to the different variants of the *MDM2* promoter was performed using the JASPAR database at http://jaspar.cgb.ki.se (Sandelin et al., 2004).

Sp1-DNA Binding Analysis

Biomolecular interaction analyses (BIA) were performed on the BIACORE T100 (GE Healthcare, BIACORE AB, Uppsala, Sweden). Series S sensor chip CM5 and amine-coupling reagents, N-ethyl-N′-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), and ethanolamine HCL (Ge Healthcare, Biacore) were used to anchor the human Sp1 protein (Active Motif). CM5 sensor chips, with carboxymethylated dextran matrix, were preconditioned through three consecutive 10 s injections of 1X HBS-EP buffer (10 mM HEPES (pH 7.4), 0.15 M NaCl, 3.4 mM EDTA, and 0.05% surfactant P20), with a flow 100 μl/min, followed by two consecutive 10 s injections of 100 mM HCl, two consecutive injections of 50 mM NaOH, and two consecutive injections of 0.5% SDS. CM5 chip flow cells were activated by 0.4 M EDC and 0.1 M NHS in a 1:1 ratio (v/v). Levels of 3000–8000 RU of Sp1 protein (20 μg/ml) were immobilized at 25°C with a flow 5 μl/min on each active flow cell. In a single binding analysis, two of the different double-stranded DNA 104 mers representing the different variants of the *MDM2* promoter with concentration ranging from 0–400 nM were injected in separate flow cells on the same chip over both the ligand (human recombinant Sp1 protein) and a reference surface (blank) for binding strength comparison. The analysis temperature was 25°C, the contact time was 60–120 s and the flow rate was 30–100 μl/min. DNA binding to Sp1-coated Chip, as opposed to reference, confirmed recombinant Sp1 to retain its activity (DNA binding capability) after being linked to the chip surface. All *MDM2* promoter fragments were compared with the wild-type variant. Biosensor data were analyzed using the BIAevaluation software version 2.0.1. Subsequently, all differences in relative binding strength were confirmed by replicates and separate pairwise analyses of all possible combinations of the polymorphic variants on the same chip (12 different experiments in total).

Cohorts for Ethnic Comparison

The different ethnic populations used for initial characterization of SNP285C distribution have been described elsewhere (for details and references, see Supplemental Experimental Procedures).

Healthy Controls Used for Cancer Risk Comparison

Norwegian healthy controls (n = 1337) were collected from several different cohorts (see Tables S1 and S2 and Supplemental Experimental Procedures). Dutch healthy controls (n = 1181) consisted of blood donors from the regions surrounding the University Hospitals of Rotterdam and Leiden, and a series of healthy individuals from all over The Netherlands (see Supplemental Experimental Procedures for further details).

Breast Cancer Cohorts

We analyzed samples from 2121 breast cancer patients from several cohorts previously described (for details and references, see Supplemental Experimental Procedures).

Ovarian Cancer Cohorts

We analyzed samples from 832 women diagnosed with epithelial ovarian cancer at the Oslo University Hospital Radiumhospitalet in the period between 2002 and 2009. The age range was 18–90 years. Prior to inclusion, pathology reports had been audited by two of the investigators (M.B. and A.D.). All patients were counseled and signed a written informed consent before genotyping. Notably, patients with known mutations related to hereditary breast and ovarian cancer (*BRCA1* or *BRCA2*) or Lynch syndromes (*MSH2*) were not included in the study.

The cohort of ovarian cancer patients used as a verification set (n = 1161) consisted women diagnosed with epithelial ovarian cancer either at Oslo University Hospital Radiumhospitalet, or Haukeland University Hospital, Bergen, in the period between 1993 and 2009. The age range was 14–90 years. Prior to inclusion, pathology reports had been audited by one of the investigators (A.D. or H.B.S.).

Ethical Considerations

All healthy individuals as well as cancer patients included in the different cohorts provided informed consent for anonymous use of the blood for scientific research. Each study included in this paper was conducted in accordance with the Helsinki Declaration. Approval for sample collection and genetic analyses on nonidentified specimens was granted by the following Regional/Institutional ethical committees: (1) Regional Ethical Committees, Health Regions of Western, South-Eastern, and Central Norway; (2) The Medical Ethical Committees of the Leiden University Medical Center, Leiden, and the Erasmus MC-Daniel den Hoed Cancer Center Rotterdam, The Netherlands; (3) The Ministry of Social Affairs and Health in Finland and the Ethics Committee of the Hospital District of Helsinki and Uusimaa, Finland; (4) The Central Manchester research ethics committee, UK; (5) The Institutional Review Board of the Chinese Academy of Medical Sciences Cancer Institute, China.

MDM2 Promoter Screening

All the Norwegian and Dutch samples were analyzed with an identical method. Thus, a region of the *MDM2* promoter containing both SNP285 and SNP309 was amplified by using the DyNazyme EXT polymerase system (FINNZYMES) according to the manufacturer's instructions with primers MDM2PF 5′-CGG GAGTTCAGGGTAAAGGT-3′ and MDM2PR 5′-AGCAAGTCGGTGCTTACC TG-3′. PCR conditions were an initial step of 94°C, 40 cycles of 94°C for

1 min, 59°C for 30 s, and 72°C for 1 min, followed by a final step of 72°C for 7 min. PCR products were sequenced using Big Dye terminator mixture (Applied Biosystems). All sequencing reactions were carried out with the same primers as used for PCR amplification. After an initial step of 5 min denaturation at 94°C, the sequencing reaction was carried out for 40 cycles of 10 s at 94°C, 5 s at 55°C, and 4 min at 60°C. Capillary gel electrophoresis, data collection and sequence analysis were performed on an automated DNA sequencer (ABI 3700). For Norwegian and Dutch samples (both for cases and controls), 2%–5% of the samples were analyzed in duplicate as assay quality controls.

Finnish and Chinese samples were genotyped by PCR-based restriction fragment length polymorphism analyses (PCR-RFLP), while British samples were analyzed for SNP309 by Taqman-assay and SNP285 by pyrosequencing (for full details of these analyses, see Supplemental Experimental Procedures).

Statistical Analysis

All statistical analyses were performed using the SPSS/PASW (version 15.0.1/17) software packages. SNP285 and 309 distributions were compared between Norwegian and Dutch healthy individuals using Chi-square test to ensure homogeneity in the cohorts used as controls (for calculations, see Table S3). For calculations of odds ratios, confidence intervals (CI) are given as 95%. For breast cancer, common odds ratio adjusted for countries (Mantel-Haenszel test) was used to ensure that the results were not affected by potential differences between Norwegian and Dutch cohorts. Ovarian cancer cases (Norwegian) were compared with Norwegian controls. However, additional analyses, including Dutch controls strengthens all calculations of the impact of SNP309G and SNP285C on ovarian cancer risk. All p-values are given as two-sided and p-values from Fischer exact tests are given as cumulative.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, and three tables and can be found with this article online at doi:10.1016/j.ccr.2010.12.019.

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