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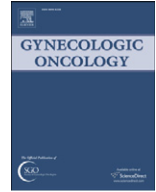

Interaction of immunological genes on chromosome 2q33 and IFNG in susceptibility to cervical cancer

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ABSTRACT

Objective. Cervical cancer is caused by persistent infection with human papillomavirus and genetic susceptibility factors may augment disease risk. The immune response consists of complex interactions and it was recently proposed that the association of combinations of genotypes at several genes should be examined. In support of this the combination CD28+17(TT)/IFNG+874(AA) was shown to increase cervical cancer risk in a Brazilian population (VB Guzman et al. New approach reveals CD28 and IFNG gene interaction in the susceptibility to cervical cancer. Hum Mol Genet 2008;17:1838–44) and our aim was to replicate this finding.

Methods. We re-examined the proposed associations by analysis of polymorphisms at CD28, IFNG, TNF, PDCD1, ICOS and CTLA4 in 1306 Swedish cases and 811 controls.

Results. Logistic regression analysis detected association at single SNP level for CD28+17 ($p=0.01$), IFNG +874 ($p=0.02$), and PDCD1+7785 ($p=0.04$). The two locus combination CD28+17(TT)/IFNG+874(AA) (OR = 0.76 (0.60–0.96, empirical $p=0.03$) and the three-locus combination CD28+17(TT)/IFNG+874 (AA)/ICOS+1564(TT) (OR = 0.65(0.49–0.87), empirical $p=0.006$) were associated with decreased risk. The strongest association was detected for the combination CTLA4-319 (CC)/IFNG (AA) (OR = 0.67(0.53–0.84), empirical $p=0.0007$).

Conclusion. The observation that these combinations of loci are associated in different populations supports their importance in cervical cancer development although the opposite directions of the effect call for clarification. The polymorphisms studied might not be the functional variants per se, but linked to those exerting a functional effect. The opposite associations in the two populations could then be explained by differences in linkage disequilibrium and population structure.

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Introduction

Cervical cancer is the second most common cancer in women worldwide [1] and infection by an oncogenic type of human papillomavirus (HPV) is a necessary but not sufficient risk factor [2]. Most HPV infections are transient [3] but women with a persistent infection of high risk HPV types have an increased risk of developing cervical cancer [4-7]. The fact that biological, but not non-biological, first-degree relatives of women diagnosed with cervical tumor have a doubled risk of tumor development clearly shows that there is a genetic predisposition to the disease [8]. Genes involved in the immune response to viral infection are natural candidates for affecting cervical cancer susceptibility and the most commonly evaluated loci are the HLA class II genes, reviewed by Hildesheim and Wang [9]. Guzman et al. [10] recently proposed that as the immune response is governed by complex interactions of many proteins, combinations of genes rather than single genes should be examined. They investigated 14 SNPs in 10 genes using three independent cohorts of cases and controls of limited size (82 cases + 85 controls, 83 cases + 85 controls and 64 cases + 75 controls) in multi-locus analysis. Guzman et al. reported that being homozygous T at position +17 (rs3116496) in the *CD28* gene and homozygous A at position+874 (rs2430561) in the interferon gamma (*IFNG*) gene was associated with an odds ratio of 2.07 (1.32–3.24). It was also suggested that the addition of a third genotype; tumor necrosis factor (*TNF*)-308 (rs1800629) homozygous G, programmed cell death 1 (*PDCD1*)+7785 (rs2227981) heterozygous CT or inducible T-cell co-stimulator (*ICOS*)+1564 (rs4404254) homozygous T might have an effect, although no significant association was found.

Loci that are located nearby on the same chromosome may be in linkage disequilibrium (LD). This means that alleles at these loci are not inherited in an independent manner but certain allele combinations occur more often than expected by random segregation. The implication of LD in association studies is that knowledge of variation at a certain position also gives knowledge of variation at linked loci. In the study by Guzman et al. the *CD28*+17 polymorphism was in LD

Abbreviations: CD28, Cluster of differentiation 28; CTLA4, Cytotoxic T-lymphocyte-associated protein 4; HLA, Human leukocyte antigen; HPV, Human papillomavirus; ICOS, Inducible T-cell co-stimulator; IFNG, Interferon gamma; LD, Linkage disequilibrium; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; OR, Odds ratio; PDCD1, Programmed cell death 1; TNF, Tumor necrosis factor.

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with a polymorphism at cytotoxic T-lymphocyte-associated protein 4 (*CTLA4*)-319 (rs5742909) and as expected there was also an association of *CTLA4*-319(CC)/*IFNG*+874(AA) with cervical cancer (OR = 1.54 (1.03–2.33)) [10].

The results published by Guzman et al. were suggestive but warrant replication as the overall number of individuals studied was quite small. The aim of the current study was to revisit the genotype combinations suggested to influence risk of cervical cancer by Guzman et al. [10] by studying polymorphisms at *CD28*+17, *IFNG*+874, *TNF*-308, *ICOS*+1564, *PDCD1*+7785 and *CTLA4*-319. Specifically, we examined the association of genotype combinations *CD28*+17 (TT)/*IFNG*+874(AA), *CD28*+17(TT)/*IFNG*+874(AA)/*TNF*-308(GG), *CD28*+17(TT)/*IFNG*+874(AA)/*PDCD1*+7785(CT), *CD28*+17(TT)/*IFNG*+874(AA)/*ICOS*+1564(TT) and *CTLA4*-319(CC)/*IFNG*+874(AA) with risk of cervical cancer development. The current investigation comprises a large material enriched for genetic susceptibility factors in order to improve the chances of detecting genetic association.

Materials and methods

The material consisted of 1306 cases diagnosed with cervical tumors (6.8% severe dysplasia, 89.6% *in situ* and 3.6% invasive cervical cancer) and 811 controls. Cases were selected from families with at least two affected women; all had a first-degree relative (mother, sister or daughter) among the cases. These families were identified by cross-linking the Swedish Cancer Registry and the National Family Registry [11]. All cases were from the Swedish population. Two sets of unrelated controls were included in order to determine allele frequencies in the general population: blood donors at Uppsala University Hospital ($n = 288$) and healthy non-obese adolescents from a Swedish obesity study [12] ($n = 523$). Among the controls, a total of 556 individuals were classified as 'Swedish' (both parents of Swedish origin or born in Sweden). The remaining 255 individuals had at least one parent born outside of Sweden or of non-Swedish origin. DNA was extracted from peripheral blood using standard methods. The study was approved by the regional ethical review board in Uppsala and all cases provided written informed consent.

Genotyping

The polymorphisms were genotyped using TaqMan genotyping assays (Applied Biosystems, Foster City, CA, US) and the 7900 HT Fast Real Time PCR system (Applied Biosystems) using Absolute QPCR ROX mix (ABGene, Epsom, UK) and 10 ng of DNA per reaction. Thermal cycling consisted of an initial step at 95 °C for 15 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Further details regarding SNP assays are available upon request. Genotypes were assigned using the SDS Software v2.3 (Applied Biosystems). For a subset of samples the polymorphisms in *TNF* and *CTLA4* were genotyped as previously described [13] using an assay developed by Roche Molecular Systems (Roche Molecular Systems, Pleasanton, CA, US) based on multiplex PCR and a reverse hybridization linear array with immobilized sequence-specific oligonucleotide probes. Genotypes were assigned by the Roche StripScan software version 5.7.1 (Roche Molecular Systems). In order to verify robustness of the genotyping, negative (no template) controls and internal reference samples were included. For the SNPs that were typed using two different methods 20 samples were genotyped using both methods to ensure consistent results. The threshold for evaluation of Hardy Weinberg equilibrium was $p = 0.01$.

Statistical analysis

Basic statistical analysis was performed in SAS 9.1.3 (SAS Institute, Cary, NC, US). The genotype distribution of all SNPs was evaluated under the additive model using the Cochran-Armitage test for trend, and the impact of genotypes at individual loci was analyzed using logistic regression. Differences in the frequency of genotype combinations between cases and controls were evaluated using Pearson chi-square statistics. Association analysis was performed comparing cases ($n = 1306$) to Swedish controls ($n = 556$) as well as to the larger group including all control individuals ($n = 811$).

The statistical power to find genetic susceptibility factors was increased by use of cases from a family material. These samples are enriched for genetic influence on cervical cancer susceptibility; i.e. they are more likely to share genetic risk factors than randomly selected sporadic cases. However, the use of related subjects may lead to inflated associations simply because of family structure. The software tool PedGenie, provided in the software Genie 2.6.3 [14,15], was used to reassess associations that appeared significant in the primary analysis. PedGenie incorporates pedigree data together with data from unrelated samples and calculates empirical *p*-values corrected for the interrelatedness of the individuals. Pearson chi-square statistics were calculated using $n = 10{,}000$ gene-drop simulations.

Haploview version 4.1 [16] was used to evaluate the linkage disequilibrium (LD) between the SNPs on chromosome 2q33 and to perform haplotype analysis. Haplotype associations were adjusted using 10,000 permutations. All samples were included regardless of family structure.

The aim of this study was to analyze polymorphisms previously suggested to be associated with cervical cancer susceptibility, why no correction for multiple testing was applied and $p < 0.05$ considered statistically significant.

Results

All SNPs were successfully genotyped in >98% of the samples. For SNPs where two methods of genotyping were used the consistency of genotyping was verified in samples that were typed with both methods yielding 100% concordant results. There was no departure from Hardy–Weinberg equilibrium for any of the SNPs. Genotype and allele frequencies for the 6 SNPs are shown in Table 1. For controls, allele frequencies did not differ between the group containing only individuals of Swedish origin and the group of all controls. At single SNP level, a trend in genotype distribution was detected for *IFNG*+874 ($p = 0.03$ in Swedish controls, and $p = 0.007$ in all controls, analysis corrected for interrelatedness of cases) and, similarly, for *PDCD1*+7785 ($p = 0.02$ and $p = 0.01$ for the Swedish and all controls, respectively). Logistic regression analysis supported the association of these SNPs and also suggested association of *CD28*+17 ($p = 0.01$).

Genotype combinations were evaluated comparing the frequency in 1306 cases and 556 Swedish controls (Table 2). The genotype combination *CD28*+17 (TT) and *IFNG*+874 (AA) was less common in cases than controls with an Odds Ratio = 0.76 (95% CI 0.60–0.96), $p = 0.02$ (uncorrected), $p_{emp} = 0.03$ (empirical *p*-value corrected for familial structure obtained by 10,000 simulations in PedGenie). The addition of a third genotype showed that the combination of *CD28*+17(TT)/*IFNG*+874(AA)/*ICOS*+1564(TT) was also associated with decreased risk with OR = 0.65 (0.49–0.87), $p = 0.004$, $p_{emp} = 0.006$). There was no association for the three-locus genotype *CD28*+17(TT)/*IFNG*+874(AA)/*TNF*-308(GG) or *CD28*+17(TT)/*IFNG*+874(AA)/*PDCD1*+7785(CT). Including also non-Swedish controls in the analysis did not affect the results for *CD28*+17(TT)/*IFNG*+874(AA); OR = 0.78 (0.63–0.97), $p_{emp} = 0.03$. For the combination of the three genotypes *CD28*+17(TT)/*IFNG*+874(AA)/*ICOS*+1564(TT) the effect remained but the result was somewhat less significant OR = 0.73 (0.56–0.94), $p_{emp} = 0.02$).

The *CD28*, *CTLA4* and *ICOS* genes are all situated on chromosome 2q33 in a region spanning 255 kb. The LD pattern was investigated using Haploview. In our material (cases and Swedish controls) there was strong LD between *CD28* and *CTLA4* (D' = 0.81, $r^2 = 0.33$, LOD = 126), less LD between *CTLA4* and *ICOS* (D' = 0.77, $r^2 = 0.02$,

Table 1
Genotype data including results of association analysis and allele frequencies for the SNPs studied.

SNP	rs number	Chromosome location	Genotype	Cases	Swedish controls	All controls	Test of association			Major allele frequency		
							p_{trend}[a]	p_{emp}[b]	$p_{log\ reg}$[c]	Cases	Swedish controls	All controls
CD28+17	rs3116496	2q33	TT	916	366	538						
			TC	343	175	253	NS		0.01	0.84	0.82	0.82
			CC	42	15	19						
IFNG+874	rs2430561	12q14	AA	354	187	274						
			AT	650	253	371	0.02	0.03	0.02	0.53	0.57	0.57
			TT	286	110	157						
TNF-308	rs1800629	6p21.3	GG	891	396	589						
			GA	340	138	188	NS		NS	0.84	0.84	0.85
			AA	32	18	27						
PDCD1+7785	rs2227981	2q37.3	CC	471	176	257						
			CT	603	258	375	0.01	0.02	0.04	0.59	0.55	0.55
			TT	226	122	178						
ICOS+1564	rs4404254	2q33	TT	798	336	473						
			TC	429	186	279	NS		NS	0.78	0.78	0.76
			CC	69	29	52						
CTLA4-319	rs5742909	2q33	CC	1044	458	666						
			CT	228	92	138	NS		NS	0.90	0.91	0.91
			TT	9	4	4						

[a] Cochran-Armitage test for trend in genotype distribution comparing cases and Swedish controls.
[b] Test for trend corrected for family structure using Pedgenie.
[c] Analysis of association by logistic regression comparing cases and Swedish controls.

LOD = 6) and not much LD between *CD28* and *ICOS* (D' = 0.25, $r^2 = 0.003$, LOD = 1). Haplotypes were constructed in order to capture variation across the three genes. There was no association of any of the most common *CD28*, *CTLA4* and *ICOS* haplotypes with cervical cancer. However, there was a slight association of the haplotype containing C at all loci which occurred in 2% of cases and 3% of controls but the low frequency of this haplotype made it difficult to draw any conclusion from the result (Table 3). The *CD28*+17 allele T was strongly linked to the *CTLA4*-319 allele C, why we also investigated the association pattern of the genotype combination *CTLA4*-319(CC)/*IFNG*+874(AA). This genotype combination was associated with reduced cervical cancer susceptibility with OR = 0.67 (0.53–0.84), $p = 0.0005$, $p_{emp} = 0.0007$. Including all controls in the analysis resulted in a similar observation, OR = 0.69 (0.56–0.84), $p_{emp} = 0.0003$.

Discussion

The current investigation assessed the influence of certain genotype combinations at loci affecting cell mediated immunity and T-cell activation on susceptibility to cervical cancer. The genotype combination *CD28*+17(TT)/*IFNG*+874(AA), previously found to increase risk of cervical cancer in a Brazilian population (OR = 2.07 (95% CI 1.32–3.24)) [10], was associated with decreased risk in our material (OR = 0.76 (0.60–0.96). Our study further demonstrates protective effects of the same genotype combination with the addition of the *ICOS*+1564 homozygous T genotype. Guzman *et al.* [10] reported that the polymorphism in *CTLA4* was in LD with *CD28* and therefore excluded this locus from their initial analysis of association. However, when they analyzed the combination *CTLA4*-319(CC)/*IFNG*+874(AA) this also showed an association, although the effect seemed smaller than that of *CD28*TT+17/*IFNG*+874(AA) [10]. Our material also displayed LD between *CD28* and *CTLA4* and the combination *CTLA4*-319 (CC)/*IFNG*+874(AA) was associated with cervical cancer, OR = 0.67 (0.53–0.84). This was the most significant association detected in our study ($p = 0.0007$ correcting for family structure and restricted to Swedish controls).

Also noteworthy is that at the single SNP level, associations were detected for the *IFNG*+874 as well as for the *CD28*+17 and *PDCD1*+7785 but not for the *ICOS* or *CTLA4* SNPs that were included in the combined genotypes showing association. These single SNP associations would not remain statistically significant if corrected for multiple testing. Taken together, the overall results of this study indicate an effect of at least one locus on chromosome 2q33 acting in combination with either *IFNG* or another gene nearby. Interestingly, haplotype analysis of the three loci on 2q33 did not result in any strong association but this might reflect the fact that the 2q33 region comprises 255 kb with limited LD. Another interesting observation was that there was no association when the combination *CD28*+17(TT)/*ICOS*+1564(TT), excluding *IFNG*, was analyzed. This supports the existence of an interaction effect.

The fact that an association of a certain combination of loci is observed in several populations supports the involvement of these loci in cervical cancer susceptibility although the opposite effects are disturbing. There are several alternative explanations to the observation that associations seem to act in opposite directions in the Swedish and Brazilian populations. Firstly, the effect of a SNP may differ depending on the genetic background and environmental factors affecting a population. It is also possible that the polymorphisms

Table 2
Frequencies of the specified combinations of genotypes.

Genotype combination	Cases (%)	Swedish controls (%)	All controls (%)	P[a]	p_{emp}[b]	OR (95% CI)[c]
CD28(TT) IFNG (AA)	251/1293 (19)	133/552 (24)	190/805 (24)	0.02	0.03	0.76 (0.60–0.96)
CD28 (TT) IFNG (AA) TNF (GG)	179/1290 (14)	92/555 (17)	138/810 (17)	NS		0.81 (0.62–1.06)
CD28 (TT) IFNG (AA) PDCD1 (CT)	132/1298 (10)	68/553 (12)	92/806 (11)	NS		0.81 (0.59–1.10)
CD28 (TT) IFNG (AA) ICOS (TT)	140/1296 (11)	86/551 (16)	115/804 (14)	0.004	0.006	0.65 (0.49–0.87)
CTLA4 (CC) IFNG (AA)	278/1286 (22)	160/549 (29)	230/802 (29)	0.0005	0.0007	0.67 (0.53–0.84)

[a] Testing for differences between cases and Swedish controls using Pearson's χ^2 statistic, no correction applied.
[b] Testing for differences between cases and Swedish controls using Pearson's χ^2 statistic corrected for family structure, empirical p-value obtained from 10,000 simulations in PedGenie.
[c] Odds ratio for individuals positive for this genotype combination, based on comparing cases and Swedish controls and no correction applied.

Table 3
Frequencies of haplotypes on chromosome 2q33.

CD28+17	CTLA4-319	ICOS+1564	Haplotype frequency			p[a]	p_{perm}[b]
			Cases	Swedish controls	All controls		
T	C	T	0.63	0.61	0.60	NS	
T	C	C	0.20	0.19	0.20	NS	
C	T	T	0.08	0.07	0.06	NS	
C	C	T	0.07	0.08	0.08	NS	
C	C	C	0.02	0.03	0.03	0.007	0.02
T	T	T	0.01	0.02	0.02	NS	

Haplotypes constructed by Haploview using all cases and disregarding family structure.
[a] p-value for association of haplotype comparing cases and Swedish controls using Haploview.
[b] p-value for haplotype association after 10,000 permutations in Haploview.

studied are not the functional variants *per se*, but merely markers in LD with the variants having a functional effect on cancer risk. Differences in LD pattern between populations could result in different haplotype patterns for the causal SNP and those assayed. The functional allele could be linked to opposite marker alleles causing seemingly opposite associations for the marker variants. Different alleles of these genes may also be important in the Swedish and the Brazilian population (allelic heterogeneity resulting in opposite association of the marker). Finally, the more limited sample size in the Brazilian study would make it more prone to type 1 errors. We conclude that the results of both studies support that IFNG and loci at 2q33 are important in cervical cancer susceptibility but more data are needed to accurately understand the effects of the allele and genotype combinations involved.

The genes discussed are all putative candidates for affecting cervical cancer susceptibility. *CD28* is a receptor expressed by T cells interacting with the B7 molecules on antigen presenting cells providing co-stimulatory signals necessary for T-cell activation [17,18]. *IFNG* is a signature cytokine of the T_H1 response and essential for immunity against viral infections and also involved in tumor control [19]. The influence of variation at the *IFNG* locus on cervical cancer susceptibility has been previously studied. A microsatellite polymorphism adjacent to the +874 SNP has been associated with risk of cervical cancer in Asian [20] and north Indian populations [21] while no association was seen in a South African study [22]. Expression of *ICOS* is critical for T-cell activation [23] and *CTLA4* is involved in negative regulation of T-cell activation [24,25].

Variation in the 2q33 region, encompassing the genes for *CD28*, *CTLA4* and *ICOS*, has been associated with autoimmunity [26-28] and progression of HIV-1 [29], but the functional variants remain to be identified. The importance of cell mediated immunity in clearance of HPV infection was highlighted by a recent review concluding that HIV infection strongly increases risk of HPV infection and squamous intraepithelial lesions [30]. This underlines the potential relevance of the 2q33 gene cluster in cervical cancer susceptibility.

The *IFNG*+874 SNP alters an NF-κB binding site and is also adjacent to a di-repeat polymorphism in the first intron of the gene that has been showed to affect expression *in vitro* [31,32]. A previous study proposed the di-repeat to be associated with risk of cervical cancer susceptibility in Taiwan, and had the surprising observation that the susceptibility genotype increased expression levels *in vitro* [20]. The increase in expression could be caused not by the repeat itself but by some variant in LD with the repeat, and the number of repeats found to correlate with high or low expression might differ between populations due to recombination. The *IFNG*+874 A allele was suggested to increase risk of cervical cancer in a North Indian [21], but not in a South African study [22]. The latter study pointed out that this polymorphism displays large frequency variation between different ethnic groups. Table 4 shows frequencies of genotypes and alleles of *IFNG*+874 in different populations. It is evident that ethnicity may confound associations unless cases and controls are properly matched. The relative frequency of the risk-associated variant in the population will also affect the impact this locus has on disease susceptibility and may provide another explanation for differing results in different populations. The frequency of AA-homozygotes among controls was quite similar in the current study and that of Guzman et al., but where the frequency in Swedish cases is lower compared to controls there is higher frequency in the Brazilian cases. Guzman et al. classified ethnicity by external phenotype characteristics but others have reported that physical appearance, such as color, is a poor predictor of genomic ancestry in Brazilians [33].

The polymorphism at +874 is an A/T SNP located adjacent to a di-nucleotide repeat of varying length; something that might cause some

Table 4
Frequencies of IFNG+874 genotypes in control individuals from various populations.

Study	Population (ethnicity if stated)	Genotype distribution			Frequency	
		AA	AT	TT	AA	A
Present investigation	Swedish	274	371	157	0.34	0.57
Guzman et al. [10]	Brazilian (white, mulatto and black)	67		126[a]	0.35	
von Linsingen et al. [37]	Brazilian (European or European/African)	15	24	11	0.30	0.54
Franceschi et al. [34]	Brazilian (mixed)	76	131	33	0.32	0.59
Matos et al. [38]	Brazilian-Rio de Janeiro area	224	273	112	0.37	0.59
Larcrombe et al. [36]	Canadian (Caucasian)	42	42	29	0.37	0.56
	Canadian (Cree)	27	14	4	0.60	0.76
	Canadian (Dené)	57	4	0	0.93	0.97
Bai et al. [35]	Chinese (Chinese)	95	37	11	0.66	0.79
Öhman et al. [39]	Finnish	69	85	21	0.39	0.64
Poli et al.	Italian (Caucasian)	116	170	77	0.32	0.55
Gangwar et al. [40]	North Indian	70	115	45	0.30	0.55
Govan et al. [22]	South African (mixed race)	158	81	26	0.60	0.75
	South African (African)	102	31	7	0.73	0.84
Tangwattanachulepoorn et al. [41]	Thai	92	53	9	0.60	0.77

[a] AT+TT, no information on genotype or allele frequencies available in paper.

confusion when comparing the results from different studies. *IFNG* remains an interesting candidate gene but the impact of variation on the functional level needs to be studied in more detail. Altered expression of IFNG would also influence susceptibility to other diseases related to infections or the immune response and this polymorphism has been studied in several such traits [34-36].

In conclusion, we have shown by analysis of specific genotype combinations that genetic variation at 2q33 in combination with variation in, or in the vicinity of, *IFNG* is associated with susceptibility to cervical cancer in Swedish women. Presently, the genotype combinations that show association should be regarded as linked to the true mediators of susceptibility. In order to identify the variants that actually modulate immune function further studies are warranted, but it is clear that the analysis of combinations of genetic susceptibility factors may unveil associations not detected when analyzing individual loci.

Conflict of interest statement

The authors declare no conflict of interest.

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