Affected sib-pair analysis of the contribution of HLA class I and class II loci to development of cervical cancer

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Cervical cancer is a multifactorial disease and infection by oncogenic human papilloma viruses represents the main environmental risk factor. Only a subset of infections becomes persistent and develops into cancer, implying that genetic susceptibility factors are needed for malignant progression. Here, we use a population-based cohort of affected sib-pairs (ASPs) to examine the role of the human leukocyte antigen (HLA) class I and class II loci in cervical cancer susceptibility. Analysis of 278 ASPs revealed significant excess genetic sharing for all three HLA class II loci studied, DPB1, DQB1 and DRB1, with the strongest evidence for DQB1 and DRB1. No evidence of excess sharing was observed for the HLA class I HLA-B and HLA-A loci. When the material was stratified on the basis of the DQB1*0602/DRB1*1501 susceptibility haplotype, carriers showed significant sharing for all loci, whereas non-carriers showed no evidence of excess genetic sharing at any of the loci. However, for the DPB1 locus there was no difference in allele frequency between carriers and non-carriers indicating that the effect seen in DPB1 is not simply due to linkage disequilibrium. Our results show that the HLA class II represents a major genetic susceptibility locus to cervical cancer in contrary to the class I that do not appear to have a significant impact on predisposition to the disease. The strongest class II effects are coming from the DQB1 and DRB1 loci, but the DPB1 locus also contributes to the susceptibility to cervical cancer.

INTRODUCTION

Invasive cervical cancer is the second most common cancer in the world among women with 471 000 cases in the year 2000 (1). Genital forms of human papilloma virus (HPV) are recognized as the major aetiological factor and are found in almost all cervical tumours (2). The virus encodes two oncogenic proteins that directly contribute to transformation of cells and HPV associated carcinogenesis (3–5). Infections are common but only a small fraction progresses into persistent infection and cancer. Since HPV is necessary but not sufficient for development of the disease, additional factors, such as host genetic susceptibility factors, have been suggested to influence the outcome of an infection. Biological first-degree relatives of women with cervical cancer have a 100% increased risk of developing the disease compared with non-biological, adoptive, first-degree relatives of women with cervical tumour (6). A hereditary component of cervical tumours has also been indicated in comparisons of twins and in relative risk studies of mothers and daughters (7,8).

Due to the pivotal role of the human leukocyte antigen (HLA) genes in the defence against viral infections they represent natural candidate genes for susceptibility to cervical cancer. Class I HLA molecules (HLA-B, -C, -A) present foreign antigens to CD8 cytotoxic T-lymphocytes (CTL), and class II molecules (HLA-DP, DQ, DR) present antigenic peptides to CD4 helper T-lymphocytes (9). The role of helper T-lymphocytes in cervical cancer development has been studied by measuring T-cell proliferative responses (10–16) or IL-2 release (17–19), but the results have not been consistent between studies. CTL responses to HPV infections are well documented (20–23), and both CD4 and CD8 T-lymphocytes appear to be part of the HPV 16 specific CTL response (24). HPV has tropism for epithelial cells, which express class I molecules. Down-regulation or complete loss of HLA class I expression has been reported in cervical carcinoma (25), and loss of expression has been correlated with tumour invasiveness and aggressive histology (26), implicating a role of HLA class I in disease progression. However, class I down-regulation may be the result of altered levels or sensitivity to cytokines instead of direct effects of viral infections (27).
Impaired class II expression (28–30) and a reduced number of Langerhans cells have been reported in cervical cancer (31,32) and in lesions due to HPV (33). Langerhans cells, which are antigen-presenting cells (APCs) in the cervix, express class II molecules, and this provides a connection between class II and cervical cancer development.

The importance of individual HLA class I loci in cervical cancer aetiology has not been widely explored, and most of the studies have focused on the class II genes, owing primarily to the availability of robust HLA class II genotyping systems. Cervical cancer has been associated with several class II alleles or antigens, such as the HLA-DQw3 antigen (34–37) and the DRB1*1501/DRB1*04 and DRB1*11 alleles (37–39). The DBB1*0602/DRB1*1501 and the DQA1*0102/DBB1*0602 haplotypes have been associated with increased risk of cervical cancer in HPV16 infected women (40,41). The DBB1*0602/DRB1*1501 haplotype was found to be associated with infection by HPV16 in cervical cancer in situ cases in a Swedish population (42). Carriers of this haplotype also had a higher HPV16 titre, and women with high viral load were prone to long-term infections (43). Protective effects of certain HLA class II alleles have also been reported. A meta-analysis showed evidence of a protective effect of the DQB1*0603 and DRB1*13 (including DRB1*1301-5) alleles in 18 of 19 studies (44).

Among the few studies evaluating the role of the HLA class I alleles in cervical carcinoma, associations to certain HLA-B alleles have been found. The HLA-B7 allele, which is in linkage disequilibrium (LD) with the DRB1*0602 and DRB1*1501 alleles, has shown an association with cervical cancer and has been proposed to be involved in the evasion of the immune system by HPV16 (45). HLA-B7 in combination with DBB1*0302 has been associated with increased risk of the disease (46,47). Studies of the class I B, C and A loci in a merged analysis of three epidemiologic studies showed no single allele at these loci to be associated with risk of cervical cancer (48), and similar results has recently been found (49). The role of the class I molecules in the disease development therefore remains uncertain.

The relationship between HLA and cervical carcinogenesis has previously been investigated using exclusively association studies. Many of the reports were based on a limited sample size and have often been performed without correction for multiple testing, leading to type I errors. Even in relatively large case control studies it is difficult to explicitly determine the contribution of individual HLA loci to disease development, due to the large number of alleles and the strong LD among loci. Studies based on other types of materials, such as familial samples, may provide additional information on the importance of individual loci or regions within the HLA complex.

### RESULTS

**HLA genotyping**

The genotype data for the ASPs were almost complete for the five HLA loci. Of the 576 individuals, 574, 576, 575, 565 and 575 were successfully genotyped for DPB1, DQB1, DRB1, HLA-B and HLA-A, respectively.

**Genetic sharing in the HLA class I and II loci**

In order to investigate the genetic sharing in the DPB1, DQB1, DRB1, HLA-B and HLA-A loci, multipoint maximum likelihood proportions were calculated on the basis of the 278 ASPs (Table 1). All of the studied loci showed maximum likelihood proportions different from the null hypothesis ($z_0 = 0.25$, $z_1 = 0.5$ and $z_2 = 0.25$), with the strongest deviation found in the DQB1 and DRB1 loci. Both single-point and multipoint maximum lod score (MLS) values were calculated for the HLA loci (Table 2). The single-point values gave evidence of excess genetic sharing for the DQB1 and DRB1 loci at the 5% level. The multipoint lod scores, using information from all of the HLA loci, were significant for DPB1 (MLS = 0.93, $P < 0.05$), DQB1 (MLS = 1.27, $P < 0.05$) and DRB1 (MLS = 1.26, $P < 0.05$) (Table 2). No significant deviation from random genetic sharing was found for the HLA-B and HLA-A loci (Table 2). The MLSs gave higher values for most markers, and this is expected since more haplotype information is used in this analysis. The multipoint analysis is considered more powerful than the single-point approach if the order of markers is known and if the correct genetic map is used. The order of markers in the major histocompatibility complex (MHC) is well known, and we used a recently described genetic map of the region on the basis of the largest number of meioses to date (50). This map was used despite the fact that our study is based only on females and the genetic map is longer in females than in males (52). Underestimating the recombination rate, as in this case when a presumed shorter map is used, we are somewhat understimating the MLS values relative to the results obtained when a longer map is used.
Genetic sharing in the ASPs stratified on the basis of susceptibility haplotype

The DQB1*0602 and DRB1*1501 alleles, which are in strong LD, have previously been shown to be associated with an increased risk of HPV 16 infection among cervical carcinoma in situ cases in the Swedish population (42). In order to examine which alleles at the DQB1 and DRB1 loci contribute most to the sharing, the material was stratified on the basis of all observed alleles in these two loci. The most frequently shared alleles within the sib-pairs were the DQB1*0602 and DRB1*1501 alleles, which were shared in 26% (72/278) of the ASPs. These alleles contributed most to the observed excess sharing in these two loci.

To study whether the genetic sharing for other HLA loci differs between carriers and non-carriers of the DQB1*0602/DRB1*1501 susceptibility haplotype, the sib-pairs were stratified on the basis of absence or presence of these susceptibility alleles. The sib-pairs had to be at least heterozygous for this haplotype in order to be classified as carriers. In total, 72 sib-pairs were carriers and 154 sib-pairs were non-carriers; 52 sib-pairs were not concordant in their susceptibility haplotype status and were therefore not included in this particular analysis. Multipoint MLS values were calculated for the five HLA loci in both carriers and non-carriers, and a permutation test was used to determine the significance of the observed MLS scores in the stratified material. Carriers showed excess sharing in the DQB1 and DRB1 loci (MLS = 11.024, P < 0.0001 and MLS = 10.973, P < 0.0001) as well as in the DPB1, HLA-B and HLA-A loci (Table 3). This finding probably reflects LD structures within the region that arise when stratifying the material on the basis of a certain haplotype. In contrary, no significant sharing was observed for any of the HLA loci in non-carriers (Table 3).

Allele frequencies in the ASPs stratified on the basis of susceptibility haplotype

To investigate whether the excess sharing observed at DPB1, HLA-A and HLA-B was due to LD with the DQB1*0602/DRB1*1501 haplotype, the material was then stratified on the basis of carrier status. This time an individual was denoted as a carrier if she was at least heterozygous for the DQ/DR haplotype, independent of the status of her sib. Both concordant and discordant sib-pairs were thus included in this analysis. The allele frequencies at the DPB1, HLA-B and HLA-A loci were compared between carriers and non-carriers (P > 0.05, with Bonferroni correction, Table 4). For the HLA-B locus the allele frequencies were significantly different between carriers and non-carriers (P < 0.001, with Bonferroni correction, Table 5). This may be due to LD among the DQB1, DRB1 and HLA-B loci. The most obvious example of an allele showing disequilibrium with the DQB1*0602/DRB1*1501 haplotype is HLA-B*0702, which was present in 41% of carriers, whereas only in 8.5% of non-carriers. For the HLA-A locus the allele frequencies were also significantly different between carriers and non-carriers (P < 0.001, with Bonferroni correction), but no single allele showed a striking difference between the groups (Table 6).

Genetic sharing in the ASPs stratified on the basis of protective alleles

There have been reports indicating a protective effects against cervical cancer development of the DQB1*0603/DRB1*1301 haplotype (44). Only 31 of the women in our study were carriers of this haplotype, and due to the lack of power, haplotype-sharing analysis was not performed. In order to calculate the frequencies of the DQB1*0603 and DRB1*1301 alleles in the material one sib from each sib-pair was randomly selected and the frequencies of these alleles were 3% (17/556). In the Swedish population, the frequency of DQB1*0603 was 14% (controls) and 10% (cases) and the frequency of DRB1*1301 was 15% (controls) and 9% (cases) (42).

DISCUSSION

Cervical cancer is a multifactorial disease, and even though the major environmental factor is known, the nature of host
Table 5. HLA-B allele frequencies in carriers and non-carriers of DQB1*0602/DRB1*1501 susceptibility haplotype

+---------------------------------+----------------+----------------+----------------+----------------+----------------+----------------+----------------+----------------+----------------+----------------+----------------+----------------+----------------+----------------+----------------+
| HLA-B alleles                      | Frequency in carriers<sup>a</sup> <br> <br>(<i>n = 402</i>) | Frequency in non-carriers<sup>b</sup> <br> <br>(<i>n = 728</i>) |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |
+---------------------------------+----------------+----------------+----------------+----------------+----------------+----------------+----------------+----------------+----------------+----------------+----------------+----------------+----------------+----------------+----------------+
| 0702                             | 0.410           | 0.085           |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |
| 4402                             | 0.095           | 0.141           |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |
| 4001                             | 0.065           | 0.115           |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |
| 5101                             | 0.060           | 0.062           |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |
| 1501                             | 0.050           | 0.110           |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |
| 0801                             | 0.050           | 0.128           |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |
| 1801                             | 0.045           | 0.015           |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |
| 2705                             | 0.032           | 0.071           |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |
| 3701                             | 0.022           | 0.022           |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |
| 3501                             | 0.020           | 0.036           |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |
| 1402                             | 0.020           | 0.010           |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |
| 1302                             | 0.017           | 0.007           |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |
| 4403                             | 0.012           | 0.025           |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |
| 5601                             | 0.010           | 0.010           |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |
| 0809                             | 0.010           | –               |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |
| 5701                             | 0.005           | 0.034           |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |
| 5501                             | 0.008           | 0.029           |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |
| 4002                             | 0.008           | 0.010           |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |
| 3901                             | –               | 0.010           |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |

Table 6. HLA-A allele frequencies in carriers and non-carriers of DQB1*0602/DRB1*1501 susceptibility haplotype

+---------------------------------+----------------+----------------+----------------+----------------+----------------+----------------+----------------+----------------+----------------+----------------+----------------+----------------+----------------+----------------+----------------+
| HLA-A alleles                      | Frequency in carriers<sup>a</sup> <br> <br>(<i>n = 402</i>) | Frequency in non-carriers<sup>b</sup> <br> <br>(<i>n = 748</i>) |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |
+---------------------------------+----------------+----------------+----------------+----------------+----------------+----------------+----------------+----------------+----------------+----------------+----------------+----------------+----------------+----------------+----------------+
| 0201                             | 0.356           | 0.384           |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |
| 0301                             | 0.211           | 0.139           |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |
| 0101                             | 0.077           | 0.150           |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |
| 2402                             | 0.070           | 0.075           |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |
| 1101                             | 0.047           | 0.041           |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |
| 3101                             | 0.040           | 0.031           |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |
| 2601                             | 0.040           | 0.015           |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |
| 2501                             | 0.035           | 0.012           |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |
| 6801                             | 0.030           | 0.044           |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |
| 3201                             | 0.030           | 0.028           |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |
| 2902                             | 0.020           | 0.025           |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |
| 2301                             | 0.012           | 0.019           |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |                 |

<sup>a</sup>Aleles with a frequency of more than 0.010 in either one or both groups.
<sup>b</sup>All sibs at least heterozygous for DQB1*0602/DRB1*1501.

The analysis of the other HLA loci indicates different relationships to cervical cancer. First, the DPB1 locus showed evidence of excess genetic sharing for the whole ASP material and in carriers of the susceptibility haplotype. No significant MLS values were found in non-carriers, but the similarity in allele frequencies between the two stratified groups suggests that the sharing observed in carriers is not simply due to LD with the DQB1*0602/DRB1*1501 haplotype. Although the strongest class II effects are coming from the DQB1 and DRB1 loci, the DPB1 locus also seems to contribute to the susceptibility to cervical cancer, even though one cannot formally rule out that the results are due to LD with other (non-HLA) genes in the region. The DPB1 alleles that might have a role in disease development are therefore likely to act in a multiplicative rather than additive mode for carriers of the susceptibility haplotype (53).

Second, the HLA-B locus showed no evidence of excess sharing in the ASPs, and the locus is therefore not likely to influence the genetic predisposition to cervical cancer. After stratification of the material, significant genetic sharing was observed among carriers of the DQB1*0602/DRB1*1501 haplotype, but not among non-carriers. These results do not support an independent effect of the HLA-B locus on cancer susceptibility. The HLA-B allele frequencies were also significantly different between the two groups of carriers and non-carriers. As exemplified by the extended DQB1/DRB1/HLA-A haplotypes, the difference between the groups of carriers and non-carriers presumably reflects LD relationships among the DQB1, DRB1 and HLA-B loci.

Third, the MLS value for the HLA-A locus, based on all ASPs, did not indicate that this locus contributes to cervical cancer susceptibility. Significant sharing was found among carriers of the DQB1*0602/DRB1*1501 haplotype, but not for the non-carriers. Yet, no allele shows appreciable differences in frequency between the two stratified groups, and no obvious extended DQB1/DRB1/HLA-A haplotypes were observed. One can therefore speculate about a possible individual role of the HLA-A locus in cancer predisposition, acting in an interactive manner with the DQB1*0602/DRB1*1501 haplotype. However, we cannot rule out that the results reflect the LD among the DQB1, DRB1 and HLA-A.

This study points to the importance of class II rather than class I alleles in cervical carcinoma. Other data argue strongly for a contribution of HLA class II instead of class I loci to cervical cancer risk (54). This can be explained by an important role of the resident APCs of the cervical mucosa and the dendritic cells such as the Langerhans cells, during HPV infection. It has been shown that dendritic cells bind and take up HPV derived virus like particles by endocytosis (55). Given the evidence for a role of the class II molecules in cervical carcinogenesis, the dendritic cells, which in contrary to the cervical epithelial cells express HLA class II molecules, might be involved in the key event of establishing a robust defence against HPV infection through antigen presentation to CD4 helper T-lymphocytes.

The DQB1*0603 and DRB1*1301 alleles have been indicated to have a protective effect against cervical cancer.
development (44). The frequency of these alleles was lower in the ASPs relative to the Swedish population frequency, supporting a protective role for these alleles (42). Interestingly, the frequency of these alleles was also lower in the ASPs relative to random cervical cancer cases from the Swedish population (42). This is expected since the women in the ASP material were selected on the basis of that they have a sib with the same type of cancer and thus more likely carry genetic susceptibility factors than random cases. This observation provides further support for a protective role of the DQB1*0603 and DRB1*1301 alleles.

The LD surrounding the genes in the human MHC complicates attempts to determine the involvement of individual loci. It is still theoretically possible that the DQB1 and DRB1 are only linked to the factor causing the increase in risk. The MHC encodes a number of immunologic proteins that may be involved in the reaction to a HPV infection. In order to identify the individual genes involved in cervical cancer susceptibility, future studies must include identification of complete MHC haplotypes and detailed knowledge of the recombination structures in the region. So far it is known that the MHC contains extended regions of strong association interrupted by hot spots of meiotic crossover (56) and that recombination rates differ considerably between individuals, and hot spots of recombination occur on an average every 0.8 Mb in the region (50). An integrated map of the MHC, based on 201 single nucleotide polymorphisms (SNPs), nine classical HLA loci and 18 microsatellites, also shows the complex haplotype patterns of the region (57). Disease associations with two or more markers located on opposite sides of a recombination hot spot could, theoretically, reflect epistatic interactions between the MHC loci. This may be applied to DPB1 in the present study, where the results suggest that an interaction between the DPB1 locus and the DQB1*0602/DRB1*1501 haplotype may influence disease susceptibility. This is supported by recent reports on recombination between DPB1 and RING3, located between DPB1 and DQB1 (50).

Cervical cancer develops as a multistep process, and several genes might influence the susceptibility to the disease, besides HLA. The ASP material represents a valuable asset in searching for additional loci affecting cancer development, using either whole genome scanning or large-scale candidate gene analyses. Identification of the nature of host factors affecting cervical cancer is crucial not only for understanding the aetiology of cervical cancer, but may also provide novel insight into the mechanisms of host–pathogen interaction.

### Table 7. Structure of the ASP material (n = 576)

<table>
<thead>
<tr>
<th>Description</th>
<th>Samples</th>
<th>Pairs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sister in a sib-pair</td>
<td>556</td>
<td></td>
</tr>
<tr>
<td>Third sister</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Mother</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Sib-pairs</td>
<td>278</td>
<td></td>
</tr>
<tr>
<td>Sib-pairs triplets</td>
<td></td>
<td>286</td>
</tr>
</tbody>
</table>

*Combination with third sister included.

### Table 8. Age distributions at diagnosis in the ASPs

<table>
<thead>
<tr>
<th>Description measurements</th>
<th>Age at diagnosis (n = 560)</th>
<th>Average age at diagnosis (n = 286)</th>
<th>Difference in age at diagnosis (n = 286)</th>
</tr>
</thead>
<tbody>
<tr>
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<td>30.1</td>
<td>3.4</td>
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<tr>
<td>Max</td>
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<td>24.5</td>
</tr>
<tr>
<td>Min</td>
<td>17.0</td>
<td>19.0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Third sister included.

Within the sib-pairs.

### MATERIALS AND METHODS

#### Subjects

The Swedish Cancer Registry contains records of all cases of cervical cancer since 1958. The reporting for in situ cancers increased in the late 1960s after population screening for cervical cancer was instituted in Sweden (58). All cases of cervical tumour, according to the criteria of the 7th revision of the International Classification of Diseases, born after 1940 and reported to the Swedish Cancer Registry before 1993 (maintained by the National Board of Health and Welfare, Sweden) were analysed against the National Family Register (Statistics, Sweden). By crosslinking these registers, families with different number of relatives diagnosed with cervical cancer were identified. Among the diagnoses, dysplasia constituted 10%, in situ carcinoma 85% and invasive cancer 5% (59). In total, 1800 ASPs, 2855 affected mother daughter pairs, 232 families with three cases and eight families with more than three cases were identified in the whole Swedish population by the use of these registers. The sib-pairs and mothers with at least one diagnosis of cervical carcinoma in situ were selected from the 1800 ASPs and were invited to participate in the study. In total, 4145 women were invited, and blood samples were obtained from 2135 participants (51%) corresponding to 644 ASPs.

For the present study, 576 individuals that correspond to 278 ASPs were selected. The structure of the material and ASP constitution among these individuals are shown in Table 7. In selecting sib-pairs for this study, the only criterion used was that the pairs with the smallest difference in age at diagnosis were favoured (Table 8). In the material, 23 (4%) women were diagnosed with invasive cervical cancer and 553 (96%) were diagnosed with cervical cancer in situ. Most sib-pairs, 79%, were concordant in their diagnosis of cervical cancer.

#### DNA extraction

DNA was extracted from 4.5 ml whole blood by standard phenol chloroform procedures using Applied Biosystems GENEPURE(tm) 341 Nucleic Acid Purification System. The concentration of genomic DNA was determined by using spectrophotometrics, and the DNA samples were diluted to 5 ng/μl in 96 well plates.
HLA genotyping

The typing of the class II HLA-DPB1, -DQB1, -DRB1 and class I HLA-B and HLA-A loci was accomplished by PCR amplification of groups of alleles using biotinylated PCR primers, followed by hybridization to immobilized sequence-specific oligonucleotide probes in a linear-array format. The method has been described previously (60). In brief, positive hybridization reactions were detected using a streptavidin-horseradish peroxidase conjugate and a soluble colourless substrate (3,3',5,5'-tetramethylbenzidine). Genotypes were determined using a computer algorithm on the basis of the pattern of sequence-specific oligonucleotide-probe hybridization.

Data analysis

The extent of the genetic sharing among the ASPs in the HLA loci was determined using the MLS values. The MLS values express deviation of identical by descent (IBD) sharing among ASPs and are calculated using a likelihood ratio method with the null hypothesis of Mendelian segregation expectations ($z_0 = 0.25$, $z_1 = 0.5$ and $z_2 = 0.25$). The MLS values can be calculated using constraints on the observed IBD probabilities so that they represent possible genetic models. According to Holmans (51), a MLS of 0.7 correspond to a $P$-value of 0.05 and a MLS of 2.3 to $P$-value of 0.001 if they are calculated using the possible triangle constraint (allowing for dominance). The Genehunter 2.1 package (61) was used to determine maximum likelihood proportions using possible triangle constraint and to calculate single-point and multipoint MLS values. The allele frequencies for each loci were determined from the available material, with the exception of the DQB1 and DRB1 loci, for which the allele frequencies for the Swedish population were available from previous work (42).

The significance of MLS scores as per Holmans (51) were not based on stratified data. To determine the significance of the observed MLS scores for non-carriers we performed a permutation test by randomly assigning ASPs and their relatives into groups of 154 ASPs. A total of 10 000 such groups were generated, and multipoint MLS scores were computed at all loci for each group. Our observed MLS values in non-carriers were then compared with the respective distribution of MLS scores generated by the permutation test. The significance of the observed MLS scores for carriers was accessed in the same way, but with 10 000 groups of 72 randomly assigned ASPs.

Contingency table chi-square test was used to compare differences in allele frequencies between carriers and non-carriers of the risk DQB1*0602/DRB1*1501 haplotype for the DPB1, HLA- B and HLA-A loci.

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