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Tumour necrosis factor microsatellite association with human papillomavirus cervical infection

Cervical cancer is the second most common cancer in the female population worldwide, and human papillomavirus (HPV) DNA has been isolated from more than 90% of these carcinomas. Immunoregulatory/antitumour

mechanisms include cytokines that interfere directly with HPV harbouring cells. Among these cytokines, tumour necrosis factor α (TNF α) is released by HPV infected cells and inhibits the growth of transformed cell lines.¹

TNF α is a proinflammatory/antitumour cytokine that is indispensable to the inflammatory response. The TNF locus contains several polymorphic areas, including five microsatellite markers—a, b, c, d, and e—which contain 14, 7, 2, 7, and 3 alleles, respectively. These microsatellites are associated with different degrees of TNF α secretion, and are related to a greater susceptibility to developing autoimmune and infectious diseases. A recent study reported several associations between TNF microsatellites and cancer, among other diseases.² However, up to now, the association of these markers with HPV and the progression to cervical intraepithelial neoplasia has only been studied by Ghaderi *et al.*,³ who focused specifically on TNF α alleles. The objective of our study was to verify the frequencies of TNF microsatellite alleles (a–e) in 106 patients with HPV cervical infection and to compare these frequencies with the results from 101 healthy controls.

In accordance with ethical norms, blood samples and cervical scrapings were collected from all patients at the time of their consultation. DNA was extracted from blood and scraped samples by the method of Higuchi.⁴ Primers and conditions for the polymerase

Table 1 Allelic distribution of tumour necrosis factor α (TNF α) microsatellites in 106 patients (n=212) and 101 controls (n=202)

Loci/alleles	Frequency	
	Patients	Controls
TNFa		
1	3	6
2	42	41
3	4	7
4	21	13
5	11	11
6	48	39
7	28	31
8	5	–
9	5	5
10	29	25
11	11	16
12	2	3
13	1	5
14	2	–
Total n	212	202
TNfb		
1	31	35
3	22	18
4	64	68
5	87	73
6	1	1
7	7	7
Total n	212	202
TNfc		
1	147	134
2	65	68
Total n	212	202
TNfd		
1	3	1
2	18	17
3	6	11
4	100	101
5	54	47
6	25	22
7	4	3
Total n	210*	202
TNfe		
1	28	32
2	2	3
3	180	167
Total n	210*	202

*One individual did not show amplification at the loci. n, number of chromosomes analysed.

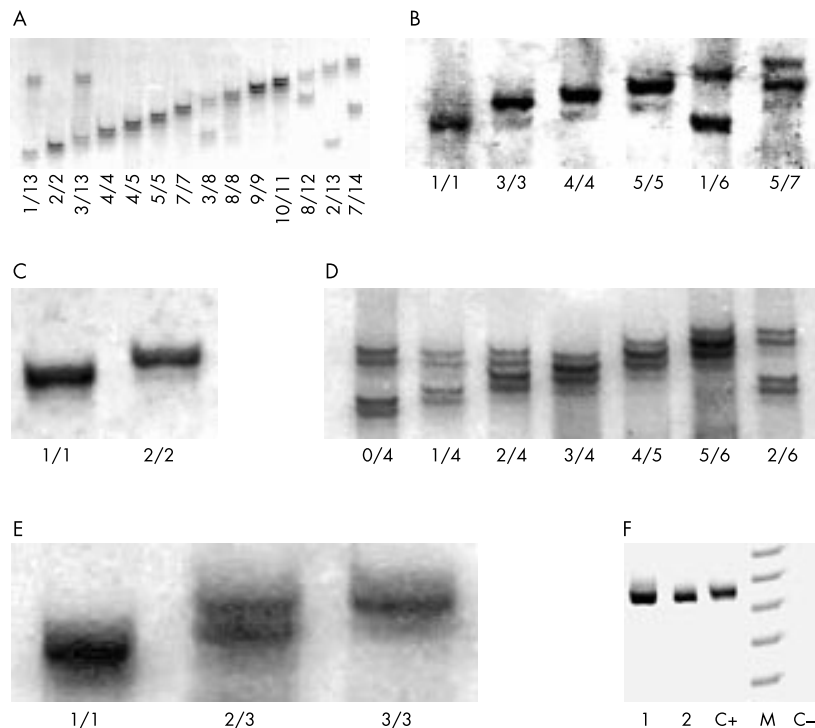


Figure 1 Polymerase chain reaction (PCR) analysis using electrophoresis on a 12% denaturing gel of (A) the tumour necrosis factor α (TNF α) allele, (B) the TNF β allele, (C) the TNF γ allele, (D) the TNF δ allele, and (E) the TNF ϵ allele. (F) PCR analysis using electrophoresis on a non-denaturing 10% polyacrylamide gel of human papillomavirus (HPV). Lane 1, patient number 1 (HPV positive); lane 2, patient number 2 (HPV positive); C+, positive control; C-, negative control; M, DNA molecular weight markers (50 bp).

chain reaction (PCR) have been described elsewhere.^{5,6} PCR products were electrophoresed on 12% denaturing polyacrylamide gels at 20 mA for TNF (a–e) and a 10% non-denaturing gel at 200 V for HPV; both gels were silver stained (fig 1). The allelic frequencies of the five TNF α microsatellite alleles were tested, for a total of 32 analyses (GENEPOP software; Population Genetics Software Package; M Raymond and F Rousset, http://wbiomed.curtin.edu.au/genepop/genepop_op1.html). No significant difference was found between the two populations (table 1). Among all the alleles, only TNF α *8

showed a significant association with HPV infection ($\chi^2 = 4.79$; relative risk, 10.73; 95% confidence interval, 1.28 to 89.9).

Our present study is the first to analyse the association of five microsatellites (a–e) of the TNF gene with cervical HPV infection, irrespective of HPV type and lesion grade. According to our results, women carrying the TNFa*8 allele had a 10 times greater risk of being infected with HPV than did the control group. In contrast with data reported by Ghaderi *et al.*,³ our analyses did not reveal an association between the TNFa*11 allele and HPV infection. Further studies are currently under way in our laboratory to determine whether the presence of specific microsatellites of the TNF gene are related to high risk HPV types or high grade cervical lesions.

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The type and quality of paraffin wax is important when constructing tissue microarrays

Tissue microarray (TMA) technology allows the representation of hundreds of tissue samples on a standard microscope slide. This is achieved by arraying small cores (0.6 mm in diameter) of paraffin wax embedded tissue samples in a recipient wax block. Sections cut from the array can then be assessed by immunohistochemistry or in situ hybridisation, according to standard protocols. TMAs enable the high throughput assessment of the presence and location of expressed genes, saving time, reagents, and clinical material. There have been several reviews on TMA construction and use,¹ but we have recently encountered a technical problem that, as far as we are aware, has not been described in the literature.

Multiple TMAs, each containing 292 cores, were constructed according to standard protocols.² Sections were cut without mishap using the adhesive tape transfer system, and arrays were stored carefully at ambient laboratory temperature for two months. However, when the arrays were then sectioned for a second time, in two of four cases, the wax block sheared off its supporting plastic cassette. Critical analysis of the problem, literature review, and consultation with TMA experts identified several possible causes for this, which are outlined below.

First, it was suggested that when the recipient blocks were made, insufficient wax was poured through the cassette, resulting in weak adhesion between the block and the cassette. However, we ensured that the mould, including the back of the cassette, was filled and that the wax level was not allowed to recede during cooling. Second, rough handling of the arrays could weaken the wax, but we do not believe that this occurred. Third, temperature fluctuations may play a role. Although arrays were constructed and stored at ambient temperature, they were cooled after every 10–15 sections cut, according to standard procedures. We no longer cool arrays during sectioning and quality remains comparable to published studies. Fourth, sections were cut along the length of the array, which may theoretically exert excessive shearing forces on the wax and increase its potential to fracture. Consequently, we now section across the width of the array. Fifth, cores may have been arrayed too deeply into the recipient block, weakening the wax at the cassette surface. Cores are now placed at least 1 mm above the cassette. Sixth, prolonged heating of wax above its melting point may compromise its quality. However, our wax is heated to its melting point of 60°C and kept molten for no longer than one week.

Finally, the quality of wax used to make the recipient block is important. The wax that we had used initially did not contain plasticisers. TMA experts and wax suppliers recommend

instead using a pliable wax containing plastic polymers, for example Precision Cut (Thermo Shandon, Runcorn, UK). Moreover, discussions with our wax supplier suggested that the quality of the batch of wax used for the recipient blocks was compromised. Other users of this batch had also reported blocks breaking during sectioning. We concluded this was the principal factor affecting our arrays. In routine histopathology, such fractures are repaired by melting the wax and re-embedding the large tissue samples. This is not possible with TMAs, however, because the hundreds of thin cores would misalign.

To solve this problem we used the following procedure. The broken array was placed into a cooled metal mould, fractured surface uppermost. Using a heating iron, the fractured surface was melted to a depth of 1 mm. A fresh cassette, prewarmed in molten wax, was applied to the array surface and the mould was filled with fresh molten wax. The wax was allowed to set thoroughly and the arrays were successfully sectioned.

In conclusion, TMAs are valuable and delicate resources. To ensure that they can be sectioned on multiple occasions, they need to be carefully constructed and stored. To avoid the problem of TMA shearing we have outlined important precautions, relating especially to the quality of wax in the recipient block. Fractured arrays can be reconstructed using the procedure outlined herein.

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