Microsatellite instability and HPV genotype in Polish women with cervical cancer

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Summary

Introduction: Human papillomaviruses (HPVs) are associated with anogenital cancer. Little is known about the prevalence of microsatellite instability (MSI) in cervical cancer. The aim of this study was to investigate the incidence of microsatellite instability in cervical cancer and to see whether there is a relation between MSI, HPV and clinicopathological characteristics in the study population. *Results:* Using three assays (pU1M/2R, GP5+/6+ and E6-nested multiplex PCR) HPV was detected in 110 out of 113 patients with histologically confirmed cervical cancer. The presence of MSI was investigated in 95 of the 113 cases using seven microsatellite loci. In total, 12 out of the 95 patients (12.6%) showed MSI. None of clinicopathological parameters showed a significant difference between microsatellite stable and MSI cases. *Conclusion:* In this population of Polish cervical cancer patients, 12.6% showed microsatellite instability. There was no correlation between MSI positivity and clinicopathological parameters and/or survival.

Key words: Cervical cancer; Microsatellite instability; Clinicopathological parameters.

Introduction

Human papillomaviruses (HPVs) are associated with various benign and malignant lesions including genital warts and anogenital cancer. Epidemiologic data show that 99.7% of all cervical cancer patients are HPV-positive if appropriate techniques are used [1]. Two Polish studies have shown a much lower HPV prevalence of 53% [2] and 70.1% [3] in cervical cancer patients. In the last study this was found despite the fact that the investigators complied with the recommendations of Walboomers *et al.* [1], i.e., fresh tissue was used for HPV detection, and HPV detection was performed using PCR primer sets directed against different regions of the HPV genome (E6/E7 and L1) [3].

Other mechanisms in carcinogenesis have been described for other tumours, such as the mutator pathway in colorectal cancer. This pathway is the consequence of defects in the DNA mismatch repair (MMR) system, and is found both in sporadic and inherited cancers [4]. DNA MMR is mediated by at least six genes; hMLH1, hMSH2, hMSH3, hMSH6, hPMS1 and hPMS2 [4-6]. The primary function of this repair mechanism is to eliminate single-base mismatches and insertion-deletion loops that may arise during DNA replication [7]. A deficient DNA MMR mechanism allows mutations to be accumulated in the genome at many times the normal rate. This is most obvious within short tandem repeats of simple sequence nucleotides termed microsatellites, already prone to mutations. Unrepaired alterations in these repeat sequences give rise to microsatellite instability (MSI). The role of MSI in the development of colorectal cancer has been thoroughly investigated. Little is known, however, on the prevalence of MSI in cervical cancer and its relation to human papillomavirus (HPV) genotypes.

The aim of this study was to investigate the incidence of microsatellite instability in cervical cancer and to see whether there is a relation between MSI, HPV and clinicopathological characteristics in this study population of Polish patients.

Methods

Tissue samples and processing

For HPV analysis material from a group of 113 patients suffering from cervical cancer and treated at the 2nd Department of Obstetrics and Gynecology, Medical University of Gdansk was available.

For MSI analysis, tissue samples were available from 95 patients with a median age of 48 years, (range 27-79 years) suffering from cervical cancer and treated between 1996 and 2001 at the above mentioned institute. Tumours with available paraffin blocks were selected from the archives of the Department of Pathology, Medical University of Gdansk. Normal tissue from the same patient, necessary for MSI analysis, was obtained from concomitant tissue resections (mainly tissue samples from the muscle of the cervix).

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HPV analysis

For HPV analysis, DNA was extracted in Poland using silica-based chromatography minicolumns (Genomic DNA Prep Plus, A&A Biotechnology, Gdansk, Poland), according to the manufacturer's recommendations. Integrity of the isolated DNA was controlled by β-globin PCR. HPV analysis in Poland was performed as described previously [3]. In brief, PCRs were carried out using the pU-1M/pU-2R and MY09/MY11 primer sets. The pU-1M/2R allows amplification of E6/E7 gene fragments of high-risk HPV types [8]. The MY09/11 consensus primer set permits amplification of HPV L1 gene [9].

All of the PCR reactions were performed in 25 µl reaction mixtures containing 2.5 µl concentrated PCR buffer (10 mM Tris/HCl, pH 8.81, 5 mM $MgCl_2$, 50 mM KCl, and 0.1% Triton X-100), 0.5 µl 10 mM dNTP, 1 µl each of the sense and antisense primers (10 µM), 0.5 µl (2 U/µl) DyNAzyme II DNA polymerase (FinnZyme, Espoo, Finland), 14.5 µl distilled water, and 80 ng (5 µl) template DNA. The amplification profile was as follows: preliminary denaturation at 94°C for two minutes, 30 cycles consisting of denaturation at 94°C for one minute, annealing at 55°C for two minutes, and extension at 72°C for two minutes, followed by a final extension at 72°C for five minutes in a programmable thermocycler (Personal Cycler; Eppendorf, Hamburg, Germany). Typing analysis on HPV-positive samples was performed on PCR amplicons by RFLP using restrictions enzymes for detection HR HPV types – 16, 18, 31, 33, 39, 45, 52b, 58.

The PCR amplicon was detected by polyacrylamide gel electrophoresis and silver-staining. In case of continuing negative results low-risk primers were used: pU-31B/pU-2R [10].

In Belgium, all samples were subjected to the GP5+/6+ HPV PCR [11]. Detection of PCR products was performed in an enzyme immunoassay format as described by Jacobs *et al.* [12], with additional primers for HPV types 26, 53, 73 and 82 as described in [13]. Finally, HPV-negative samples in both Poland and Belgium were analysed by the E6 NM-PCR in Germany [14].

DNA extraction from formalin-fixed, paraffin-embedded tissues

Paraffin sections were processed to extract DNA by two washes with xylene (Merck, Darmstadt, Germany), two washes with absolute ethanol (Merck) and a final wash with acetone (Merck). Inbetween washes, samples were centrifuged at 12,000 rpm for 5 min, and supernatant was carefully removed. Finally, the tissue was dried for 30 min at 50°C. To the deparaffinized dried tissue, 150 µl TE buffer containing proteinase K (200 µg/ml, Invitrogen, Merelbeke, Belgium) was added. After overnight incubation at 37°C, the lysates were boiled for 10 min, centrifuged (5 min, 12,000 rpm) and stored at -20°C until use.

MSI analysis

Primers for the following microsatellites were used: D3S1300, D3S4103, D5S346, BAT25, BAT26, CAT25 and SEC63, for details see Table 5. Forward primers were synthesised with a fluorescent tag (FAM or HEX) on the 5' end, and were purified by standard high-performance liquid chromatography (Eurogentec, Sart Tilman, Belgium). DNA (5 µl) was amplified in a 50 µl reaction, which contained 5 µl of 10 x buffer (Applera, Foster City, CA), $MgCl_2$ as appropriate (Table 3), 0.4 µmol/l primer pairs, 250 µmol/l deoxynucleoside triphosphate, and 2.5 units of AmpliTaq Gold DNA polymerase (Applera, Foster City, CA). Amplifications were performed using a 2-min initial denaturation at 94°C, followed by 30 cycles of 45 sec at 94°C, 45 sec at the annealing temperature, and 45 sec at 72°C, and 5 min final extension at 72°C in a Hybaid PX2 thermocycler (Thermo Electron, Brussels, Belgium). Amplification was checked by electrophoresis on 2% agarose gel. PCR products were then mixed with a standard size (GeneScan 400HD ROX, Applera, Foster City, CA), electrophoresed on an automatic ABI3100 DNA analyser, and evaluated with GeneScan software (Applera). Microsatellite instability was defined as the appearance of novel alleles in the tumour tissue compared to normal tissue. The lesions were categorised as microsatellite stable (MSS) if no regions of instability were detected, and MSI, if one or more of the regions showed instability.

Statistical analysis

Correlation of microsatellite instability with clinico-pathological parameters was analysed using Chi square, or with Fisher's exact test if one or more cells had a count of less than 5. For survival analysis a Kaplan-Meier curve was fitted. Significance of the difference between MSS and MSI was determined by the Mantel-Cox log-rank test.

Results

HPV detection

Among 113 patients with histologically confirmed cervical cancer, high-risk HPV DNA was found in 85 cases (75.2%) by pU1M/2R and MY09/11 PCR. In 28 (24.8%) HPV DNA was not observed. For verification of the type of HPV in positive patients, PCR-restriction fragment length polymorphism (RFLP) analysis was done. HPV detection by GP5+/6+ on all 113 samples showed HPV DNA in 85 cases. Of these, 77 were HPV positive in both assays, and eight were positive in one assay and negative in the other, and vice versa. On the remaining 20 HPV negative samples E6-nested multiplex (NM) PCR was performed. Seventeen further samples were shown to contain HPV, leaving three HPV-negative cases. The HPV genotypes found by combination of all detection techniques are shown in Table 1.

Table 1. — *HPV genotype distribution in cervical cancer patients.*

Single infections	no.
HPV 16	80
HPV 18	3
HPV 39	1
HPV 45	3
HPV 56	1
HPV 59	1
Multiple infections	**no.**
HPVs 16+18	6
HPVs 16+18+45	1
HPVs 16+31+35	1
HPVs 16+33+58	2
HPVs 16+35	1
HPVs 16+39+45	1
HPVs 16+45	2
HPVs 16+52	2
HPVs 16+66	1
HPVs 18+45	1
HPVs 31+35	1
HPVs 33+58	1
HPV negative	3

Table 2. — *MSI detected in cervical cancer patients per marker.*

Patient no.	Microsatellite marker D3S1300	D3S4103	D5S346	BAT25	BAT26	CAT25	SEC63
2	n.a.	–	+	–	–	–	–
8	n.a.	n.a.	+	–	–	–	–
14	n.a.	+	–	–	–	–	–
27	n.a.	–	n.a.	+	–	–	–
58	–	+	+	–	–	–	–
61	+	–	–	–	–	–	–
63	n.a.	n.a.	+	–	–	–	–
80	n.a.	n.a.	+	–	–	–	–
83	+	–	–	–	–	–	–
84	n.a.	n.a.	+	–	–	–	–
88	–	–	+	–	–	–	–
95	–	–	–	–	–	+	–

Detection results: – = microsatellite stable, + = microsatellite instable, n.a. = not amplifiable.

Table 3. — *Correlation of MSI with the presence of HPV.*

	MSI negative no.	%	MSI positive no.	%
HPV negative	3	3.8	0	0.0
HPV multiple infection*	15	18.8	1	8.3
HPV type 16	54	67.5	11	91.7
HPV (other high risk type)	8	10.0	0	0.0
Total	80		12	

* HPV 16 was found in 13 of the 16 multiple infections.

Table 4. — *Bivariate correlation of MSI positivity with clinicopathological parameters.*

	MSI positive n/evaluable	%	p value	Test*
Lesion size (largest diameter in cm)				
< 4	6/42	14.3	0.804	P
≥ 4	6/48	12.5		
Depth of stromal invasion (in mm)				
< 3	10/79	12.7	0.576	F
≥ 3	1/6	16.7		
Vascular invasion				
negative	12/85	14.1	1	F
positive		0/5	0.0	
Vaginal infiltration				
negative	7/55	12.7	0.748	P
postive	5/33	15.2		
Lymph node status				
negative	5/59	8.5	0.061	P
postive	7/31	22.6		

* Statistical test used: P = Pearson chi square, F = Fisher exact test.

Table 5. — *Microsatellite markers used for MSI detection.*

Name*	Sequence	Type**	Amplicon size
d3s1300fw	agctcacattctagtcagcct	di	225
d3s1300rev	gccaattccccagatg		
d3s4103fw	ttctactgcaatccagcctgg	di	120
d3s4103rev	gccttgggtagatttatacct		
D5S346fw	actcactctagtgataaatcg	di	115
D5S346 rev	agcagataagacagtattactagtt		
Bat25fw	tcgcctccaagaatgtaagt	mono	120
Bat25rev	tctgcattttaactatggctc		
Bat26fw	tgactacttttgacttcagcc	mono	115
Bat26rev	aaccattcaacattttaacc		
sec63fw	agtaaaggacccaagaaaactgc	mono	100
sec63rev	tgcttttgtttctgttgctttg		
cat25fw	cctagaaacctttatccctgctt	mono	150
cat25rev	gagcttgcagtgagctgaga		

*fw = forward, rev = reverse; **Type: di = dinucleotide marker, mono = mononucleotide marker.

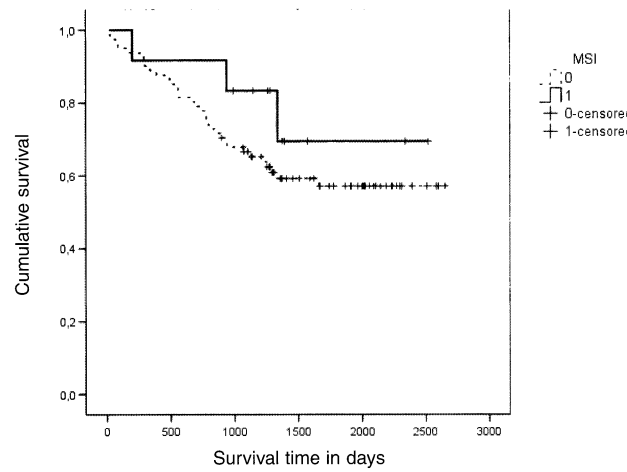

Figure 1. — Cumulative survival in MSI and MSS cervical cancer patients.
Solid line = microsatellite unstable cases, dotted line = microsatellite stable cases. Dashes represent censored cases.

MSI detection

Regrettably, tissue blocks could only be retrieved from 95 of the 113 cases. The presence of MSI in these 95 cases was investigated using seven microsatellite loci; four mononucleotide and three dinucleotide markers. Two dinucleotide markers, D3S1300 and D3S4103, generated appropriate results only in a subset of samples.

In total, 12 out of the 95 patients (12.6%) showed microsatellite instability, but only one patient showed instability at two loci (Table 2). This is at the threshold (2/7, 28.6%) of high microsatellite instability (MSI-H) as defined by the International Workshop on Microsatellite Instability [15], which states that ≥ 30% of markers need to be involved for MSI-H. However, this definition was developed for the purpose of MSI detection in colorectal cancer. As yet, it is unclear whether delineation of MSI into MSI-H and MSI-L is appropriate in cervical cancer.

Using the four mononucleotide markers, only two cases of instability were found, whereas the three informative dinucleotide markers detected 11 cases of instability.

MSI, HPV, clinicopathological parameters and survival

We studied whether MSI was associated with the presence of HPV. For this, HPV was recoded in the following categories: HPV negative, multiple infection, HPV16, other HPV types (Table 3). All MSI positive cases were HPV16 positive, the one MSI positive multiple HPV infection also included HPV16. However, this did not differ significantly from the prevalence of HPV 16 in microsatellite stable (MSS) cases.

Clinicopathological parameters available for analysis included lesion size, depth of infiltration, the presence of

vascular invasion, vaginal infiltration and the presence of lymph node metastasis (Table 4). None of these parameters showed a significant difference between MSS and MSI cases. Finally, MSI status was analysed in relation to outcome of disease (death). In the group of MSI-negative patients, 33/81 (40.78%) died of disease, whereas in the MSI-positive group three of 12 patients died of disease (25%). This difference was not statistically significant (Fisher exact, p = 0.357). Although the Kaplan-Meier curve suggested an improved survival for MSI-positive cases (Figure 2), with medians of 2029 days (MSI) versus 1822 days (MSS), this difference was not statistically significant.

Discussion

MSI was first reported in cervical cancer by Han *et al.* [16], who found two MSI positive cases out of a total of 13 samples.

Larson *et al.* [17] showed that dinucleotide repeats were much more prone to MSI than tri- or tetranucleotide repeats. In our study, we did not investigate tri- or tetranucleotide repeats, but our results suggest that dinucleotide repeats are also more prone to MSI than mononucleotide repeats. This is in contrast with the situation in colorectal cancer, since for that tumour type it has been suggested to replace the dinucleotide repeats in the Bethesda panel by mononucleotide repeats because of the higher sensitivity for MSI of the latter [18]. Both Ou *et al.* [19] and Chung *et al.* [20] showed that Bat 26 in particular is not a valid marker for MSI detection in cervical cancer. This is in accordance with our results, since no Bat 26 MSI was found.

The incidence of MSI in cervical cancer is controversial, with reports of both high incidences (ranging from 23 to 35% [20-23]) and low incidences, around 5% [17, 19, 24, 24]. Our results, with an incidence of 12.6% seem to fall inbetween these groups, but are in agreement with the results of Edelmann *et al.* [26], who found 11%. Differences in incidence could have a geographic background, but more likely reflect differences in use of markers, with respect to both the number of markers used and in the location of the markers on different chromosomes.

So far, none of the reports on MSI in cervical cancer have looked at any correlation with clinicopathological parameters. The population size of our study was limited, and this limited the power of the investigation. However, one association with clinical findings was rather surprising. Of all parameters investigated only the presence of lymph node metastasis showed a trend, as it was shown to occur nearly twice as often in MSI positive patients (7/12, 58.3%) than in MSS patients (24/78, 30.8%, p = 0.061 by Chi-square analysis). Since tumour spread to the regional lymph nodes is a strong indicator of poor prognosis, the finding of a slightly better survival of MSI positive patients would seem contradictory. However, again we realize that this observation is open for discussion due to the limited size of our study population. Moreover, the difference in survival was not statistically significant (p = 0.328).

Conclusions

Firstly, using multiple HPV detection methods, the incidence of HPV in cervical cancer was not as low as initially expected. Secondly, large differences in the incidence of MSI in cervical cancer have been reported. Although this could probably be remedied by establishing a marker panel, as has been done for colorectal cancer, there seems to be no urgent need to do so, as there is little, if any, correlation between MSI positivity and clinicopathological parameters and/or survival.

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