Evaluation of DNA mismatch repair system in cervical dysplasias and invasive carcinomas related to HPV infection

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Summary

The aim of this study was to answer the question whether the products of hMSH2 and hMLH1 genes take part in the mutation track of cervical carcinoma.

Methods: IgG1 monoclonal antibodies (Pharmingen) detecting epitopes characteristic of hMLH1 and hMSH2 were used in the present study. The value of the half-quantitative H-score coefficient was calculated. Its threshold value was 0.4. Identification of 16 and 18 HPV types was performed by PCR.

Results: An intensified hMLH1 protein expression was observed both in the squamous epithelial carcinomas and cervical adenocarcinomas (H- score of 1.44 and 0.98, respectively) as compared to the control (H-score of 0.9). However, a decreased expression of hMSH2 protein was observed in the analysed cases of carcinoma (0.9 and 0.7) as compared to the control group (1.2). An intensified expression in G3 for hMLH1 and higher hMLH1 in comparison to hMSH2 was observed.

Conclusions:

1. A considerable expression of hMLH1 and hMLH1 proteins was observed in the tissues with invasive cervical carcinoma not only within epithelial but also in stromal cells.
2. More intense expression of hMLH1 and hMSH2 was observed in invasive carcinomas and CIN than in the non-neoplastic cervical tissue lesions (erosion).
3. A stronger expression was observed for the hMLH1 than for the hMSH2 proteins - contrary to the cases of carcinomas of the uterine corpus and endometrial carcinoma.

Key words: Cervical carcinoma; HPV 16 and/or 18; Mismatch repair; hMLH1, hMSH2.

Introduction

Products of proto-oncogenes, suppressor genes and proteins which are responsible for repair of DNA mismatches during replication can play an essential role in carcinogenesis [1, 2, 3]. It is believed that if mutations have taken place, then undoubtedly the DNA repair system does not function correctly, and the risk of cancer depends on the number and location of mutations which occurred in the genome [4].

Recently it has been established that viral DNA can lead to carcinogenic transformation of a somatic cell, and it was recognized that HPV *(human papilloma virus)* can be involved in uterine cervix carcinogenesis [5]. In most cervical carcinomas (70-95%) viral DNA can be found integrated with the host cell genome [5]. In cervical cancer cells the oncogenic effect of HPV is, at least partially, the consequence of the influence of the unique viral gene *E6* and *E7* products on the fundamental regulators of the cell cycle [5]. It was revealed that the E6 protein binds to the p53 protein, and the E7 protein with the RB protein, respectively [6].

HPV-positive cervical cancer cells frequently contain an intact p53 protein, however *p53* mutations that are recognized as the most frequent changes in a large number of human cancers, are observed in only up to 6% of cervical carcinomas [5]. On the other hand, p53 protein binds to the promoter region of the hMLH1 gene and results in hMLH1 protein synthesis activation. A database search (Medline) identified the only study on hMLH1 gene structure and function in cervical carcinomas which was carried out by Hu X *et al.* [7]. The study proved that even though heterozygosity was lost in 43% of the neoplasms, expression of the hMLH1 gene was regular. Possible change of ubiquitisation quality of p53 protein in HPV positive carcinomas could also play a role in the cancer pathway. Arrangement of hMLH1 and hMSH2 proteins in cervical carcinogenesis development still has to be evaluated [8].

It was well recognized that no IHC staining of hMSH2 truncated protein occurred when tumours contained a hMSH2 mutated gene [9]. The same results were obtained by Leach *et al.* [10], in human malignant tumours using monoclonal antibodies against carboxy-terminal epitopes. These epitopes are usually lost or inactivated in tumours. Contrary to this, strong hMLH1 protein expression was observed in endometrial cancer, however these data are based on a low number of cases and now are under further invesigation [11].

Revised manuscript accepted for publication Janaury 4, 2002

Decreases in MMR gene expression is due not only to mutations observed by Katabuchi [9] and Leach [10] but also to altered methylation of the hMLH1 promoter region [11]. As cervical cancers present low p53 gene mutation frequency, the expected hMLH1 protein expression should be diminished in this type of cancer.

The fact that occurrence of HPV with a high oncogenic potential does not automatically cause cervical carcinoma seems to confirm that epigenetic influences can bring about special sensitivity in certain women to the changes caused by a long-term infection with HPV [11].

The aim of this study was to evaluate the influence of HPV infection on MMR protein expression (hMSH2 and hMLH1) in combination with a clinical evaluation of HPV-positive cervical carcinomas.

Study material

The study material included tissue samples of 41 cervical carcinomas, and 15 samples of glandular erosions obtained from patients with the III cytological smear according to Papanicolaou. Patients were aged from 24 to 62 years of age (mean age 51.3 ± 2.8). All patients were surgically treated at the I Institute and Clinic of Surgical Gynaecology of the Lublin Medical Academy and at the I and II District Hospital of Gynaecology and Obstetrics in Lublin. The weight of the tissue sections was 0.03-0.4 g.

The control group consisted of regular uterine cervix tissue obtained from 11 women (40-72 years of age) who underwent surgery due to uterine myomas. The patients were divided into groups with respect to the evaluated histopathological or clinical parameters. In the group of 41 women with cervical carcinoma, *carcinoma in situ* was diagnosed in 16 patients, invasive carcionoma in 25, of which 19 were diagnosed as suffering from *carcinoma planoepithelial* and six from *adenocarcinoma* (Table 1).

As far as histopathological type is concerned, in the group of patients with squamous epithelial carcinoma there were 12 cases of *macrocellularae keratotodes*, five cases of *macrocellularae akeratodes* and two cases of *microcellularae akeratodes*. In the group of six patients with adenocarcinoma colli uteri, *endocervicalae mucocellularae* was recognised in three cases, whereas *endometroides, clarocellularae* and *serosum papillare* were idenrtified in one case each.

With respect to dedifferentiation of the neoplastic cells, the following groups of patients were distinguished: (G1 n = 9, G2 n = 6, G3 n = 10), according to the WHO grading (Table 1).

According to the FIGO clinical staging, 16 patients were classed as stage 0 *(carcinoma in situ)*, 13 women as IA, ten as IB, and two also as IIA stage (Table 1).

Methods

Tissue samples were fixed in the 10 % buffered formalin. Then, paraffin blocks were formed and cut into sections 4 μm thick, which were then placed on glass coated with 2% solution of trimetoxysilane in acetone.

Deparaffination of the sections for DNA HPV analysis by PCR *(polymerase chain reaction)* and immunohistochemical methods was carried out by placing the sections, in turn, in xylene, absolute ethyl alcohol, 70% ethyl alcohol, and in PBS for the immuno-histochemical studies.

PCR (polymerase chain reaction)

DNA was isolated using a Nucleon ST kit (Amersham) according to the manufacturer's instructions. Purity of the obtained DNA was controlled by spectrophotometry and electrophoresis in the agarose gel. Identification of HPV types 16 and 18 was carried out in the Department of Molecular Virology, Adam Mickiewicz University in Poznań (Poland). PCR was applied with sets of primers (Table 2). The final mixture contained 1 μM of primers, 200 μM deoxynucleotide triphosphates, 1x PCR buffer (10 mM Tris-HCl_2, pH: 8.3; 50 mM KCl; 3.5 mM MgCl2 200μM deoxynucleotide triphosphates; *1U/25 μe* of Tag polymerase mixture. The samples were amplified for 30 cycles in a thermal cycler (Biometra). Each cycle consisted of the following steps: denaturation at 95°C for one minute, annealing at 59°C for 30 seconds, followed by the primer extension at 72°C for one minute. After amplification, the PCR products were analysed by Southern blot hybridisation with a ^{32}P-labelled probe specific for HPV 16 and 18 [31].

ICC (immunohistochemical studies)

Monoclonal antibodies (Pharmingen) of the IgG_1 class that detect epitopes characteristic of the hMLH1and hMSH2 proteins, were used in the gene expression analysis of the DNA

Table 2. — *Primers for PCR study*

Primers	Region of amplification	Sequence 5'-3'	Product size
MY09	L1	CGTCCMARRGGAWACTGATC	450 bp
MY11		GCMCAAGGWCATAAYAATGG	
		M=A+C,R=A+G,W=A+T, Y=C+T	
HPV16/L1A	L1	GCCTGTGTAGGTGTTGAGGT	264 bp
HPV16/L1B		TGGATTTACTCCAACATTGG	
HPV18/L1A	L1	GTGGACCAGCAAATACAGGA	162 bp
HPV18/L1B		TGCAACGACCACGTGTTGGA	
HPV18ME12	E6	CACGGCGACCCTACAAGCTACCTG	404 bp
HPV18ME50		TGCAGCACGAATTGGCACTGGCCTC	

Table 1. — *The studies groups of patients with cervical carcinoma*

FIGO Classification	n	WHO Classification	n	Histology of cancer			
				Planoepithelial	n	Adenocarcinoma	n
O	16						
IA	13	G1	9	Macrocellular keratodes	12	Mucocellular	3
IB	10	G2	6	Macrocellular akeratodes	5	Endometroides	1
IIA	2	G3	10	Microcellular akeratodes	2	Clarocellular	1
						Serosum papillare	1
				Sum	19	Sum	6
Total	41	Sum	25		Sum		25

n = number of patients

hMLH1and hMSH2 repair system. Standard IgG_1 class antibodies were used as a negative control. The positive controls were the tissue sections from the regular small and large intestine. Regular expression of the hMLH1and hMSH2 proteins was established on the basis of the H-score values for the control group of HPV negative tissues. After deparaffination, the hydrated sections were placed twice for seven minutes in a micronave oven at a power of 700 W, then they were cooled down for 20 minutes at room temperature. Endogenic peroxidase was blocked with a 3% solution of hydrogen peroxide in 100% methanol for 30 min. After PBS rinsing, non-specific binding sites were blocked with 2% normal horse serum, then the first antibodies with a concentration of 10 μg/ml were placed there and the sections were incubated over. night at a temperature of 4°C in a humid chamber. After PBS rinsing, the second biotinylated antibodies were placed and next complex avidine DH-horse-radish peroxidase-H was added. Afterwards, incubation was carried out at room temperature for 30 minutes. Colour staining was done in the PBS solution with 0.1% DAB *(3,3'diaminobenzydine tetrahydrochloride)* and 0.02% hydrogen peroxide and 0.0068% imidazol. After 5-7 minutes, the sections were stained for contrast with hematoxylin and coverslipped. The value of the half-quantity H-score coefficient [12], was calculated taking into consideration the number of stained cells expressed in percentage, and intensity of staining in the scale from + to +++. The coefficient value ranged from 0 to 3. The H-score of 0,4 was arbitrarily assumed as a threshold value.

The frequency rate of a given feature was given in the statistical analysis.

Results

Infections with HPV type 16 and/or 18 was observed in three women from the control group (3/11-28%), whereas in the group of patients with glandular erosion in five cases (5/15-33.3%). HPV virus was detected in in 14 out of the 16 patients (87.5%) with *"ca in situ"*, in 18 out of the 19 patients (95.4%) with squamous cervical carcinoma, and in all of six of the adenocarcinoma patients (100%) (Table 3).

The value of the H-score coefficient for the hMLH1and hMSH2 proteins was assumed as regular if it was above 0.4 in the analysed sections of regular tissue. The 0.4 H-score was taken as the lowest found for the regular tissues. The calculated range of the H-score for the unchanged cervix was 0.4 to 2.1. The mean H-score for the control tissue sections was 0.9 for the hMLH1protein and 1.2 for the hMSH2 protein (Table 4). The H-score for large intestine tissue was 2.6, and for small intestine tissue 2.5. These values were higher than the maximum values obtained for the regular HPV-negative uterine cervix tissues.

Table 3. — *Frequency of DNA HPV occurrence in the studied groups of patients*

Group	n	HPV 16 and/or 18 (positive)	HPV 16 and/or 18 (negative)
Control group	11	3 (28%)	8 (72%)
Erosion	15	5 (33.3%)	10 (66.7%)
Ca in situ	16	14 (87.5%)	2 (12.5%)
Ca planoepithelial	19	18 (95.4)	1 (4.6%)
Adenocarcinoma	6	6 (100%)	0

n = number of patients

Table 4. — *Comparison of individual clinical parameters and expression of proteins from the MMR system expressed as the mean values of the H-score coefficient in the examined groups of patients*

	N	hMLH1	hMSH2
Control group	11 (3)*	0.9	1.2
Erosion	15 (5)*	0.62	0.62
Differentiation of the neoplastic process according to the WHO classification			
O°	16 (14)*	0.68	0.81
I°	23	1.1	0.9
II°	2	1.4	0.7
Tumour differentiation (WHO)			
G1	9	0.9	0.6
G2	6 (5)*	0.8	0.7
G3	10	1.5	1.1
Differentiation of the neoplastic process according to the histopathological diagnosis			
Carcinoma planoepitheliale	19 (18)*	1.44	0.9
Adenocarcinoma	6	0.98	0.7

* DNA HPV analysis for 16 and/or 18 positive
n = number of patients

In the group of sections obtained form the patients suffering from epithelial and glandular carcinomas, an increased average level of the H-score was observed for the hMLH1protein. The mean value of the H-score for the hMLH1 protein was 1.44 in the cases of squamous epithelial carcinoma, and 0.98 in the cases of glandular carcinomas. The H-score for the hMSH2 protein in both types of cancer was lower, presenting 0.9 and 0.7 values respectively (Table 4).

The higher levels of the H-score for the most dedifferentiated G3 tumours were noted (1.5 for hMLH1, 1.1 for hMSH2) as compared to the tumours with low or medium dedifferentiation (0.8 for hMLH1 and 0.7 for hMSH2 in G2, 0.9 for hMLH1 and 0.6 for hMSH2 for G1) (Table 4).

The immunohistochemical results showed that stage I and II of disease (FIGO classification), are accompanied by an intensified expression of hMLH1 protein expression (H-score: 1.1 and 1.4, respectively), and lowered expression of the hMSH2 protein (H-score: 0.9 and 1.1, respectively) as compared to the control group values. In clinical stage O a lowered MMR protein expression was observed with the H-score of 0.68 for hMLH1 and 0.81 for hMSH2 (Table 4).

Comparison of hMLH1 and hMSH2 protein expression showed that in cases of cervical carcinomas combined with HPV infection, hMLH1 expression was higher than that of hMSH2. The H-score values obtained for both proteins in the tissues with diagnosed clinical stage O remained at a similar level (Table 4).

Discussion

In the studied cervical cancer tissues, the PCR detected frequency of HPV type 16 and 18 infection, was no different from the frequency stated by other authors both in the examined patients and controls using this same type of methodology.

However the estimated frequency of HPV DNA in the group of patients with CIN III (87.5%), was slightly higher than the results obtained by Bosch [13] for Spanish women with a high degree of dysplasia. Bosch proved that infection with DNA HPV was 72.4%, whereas latent infections by HPV occurred in 4.7%. Becker [14] found that in American women with CIN III, the frequency of HPV infections was 93.8%, while in patients from the control group it was as high as 42.1%. Olsen [15] showed that in Norway, HPV infections were found in women with CIN III in 90.8%, whereas in 15.4% in the control group. A meta-analysis of occurrence of DNA HPV infections in epithelial cervical carcinoma carried out by Walboomers [16], showed that HPV was present in 84-100%. It can be clearly seen that application of more sensitive methods for HPV detection, such as PCR, proved that DNA HPV was present in almost 100% of cases of cervical carcinoma.

Studies carried out in the framework of the International Biological Study on Cervical Cancer pointed to the presence of DNA HPV detected by the PCR method in 95% of the 45 glandular cervical carcinomas [17]. Whereas Ferguson [18] identified DNA HPV in only 16 (59%) out of 27 cases of the same type of cancer. The above results unambiguously show that the dominant type of HPV most often found in cases of cervical carcinoma is type 18. In our group of glandular carcinomas a 100% presence of HPV DNA probably results from the small number of cases studied and a very sensitive method using PCR.

As can be gathered from the available databases (Medline), mutation in the genes of the MMR system are the most frequent subject of studies on colon cancer neoplasias, and less frequently in endometrial cancer. Studies on the MMR, hMLH1 gene structure and function in the cases of cervical carcinomas, carried out by Hu X *et al.* [7], proved that even though heterozygosity was lost in 43% of the neoplasias, expression of the hMLH1 gene was regular. On the contrary, a decrease in MMR gene expression observed by some authors was probably due to not only mutations but also irregular hypermethylation in its promoter region [8].

In our studies, it was surprising that the hMLH1 protein content in the group with cervical carcinoma was higher than the in the control group or even in the cases with erosio glandularis. Additionally expression of hMLH1 was higher than hMSH2. Expression of hMLH1 in the cases of cervical carcinoma was higher than in the endometrial cancer detected by Ronnett [19]. However, the hMSH2 protein content was comparable to the expression detected in the tissue of the endometrial cancer [19].

The results showed higher values of the half-quantity H-score for the hMSH2 and hMLH1 proteins for the most dedifferentiated tumours (G3) as compared to the most graded tumours (G1). It could be that the disturbed metabolism in the neoplastic tissue is attempting to return to the normal pathway but it is no longer possible. In some cases, despite considerable morphological changes, the neoplastic tissue may activate repair processes with maximum intensity, difficult to find in regular conditions, sometimes at a late stage of carcinoma development. The fact that the studied G3 group of patients was of lower average age could increase the efficiency of DNA repair. However, it is very difficult to draw any precise conclusions with respect to MMR expression and its relation to histologic tumour type.

An overexpression of both proteins (hMLH1 and hMSH2) in the stromal cells was also observed in the tissues with histopathologically diagnosed cervical carcinoma. It may be that the cervical stroma triggers the system of DNA repair with an unmatched intensity.

Intensified expression of hMLH1 proteins in the analysed cases of cervical carcinoma is difficult to explain. As mentioned, p53 protein as an activator of the hMLH1 gene promoter should result in a lowered and not increased hMLH1 expression due to ubiquitisation of p53 proteins in HPV positive carcinomas. The reduced expression of hMSH2 proteins can, perhaps, be explained by the above mechanism. Different expressions of hMLH1 and hMSH2 proteins suggest not only a different mechanism of their activation but also different DNA repair protein function in the HPV positive cervical carcinomas.

The reasons for the irregular functions of the MMR genes have not yet been fully explained, hower the results of the immunohistochemical analysis of the hMSH2 and hMLH1 proteins in cervical carcinomas suggest that these latter proteins can be successfully used as a preliminary marker probably useful for the histopathological dedifferentiation of cancer cells.

Conclusions

1. A considerable expression of hMLH1 and hMLH1 proteins was observed in the tissues with invasive cervical carcinoma not only within the epithelial cells but also in the stromal cells.
2. More intense expression of hMLH1 and hMSH2 was observed in invasive carcinomas and CIN than in the non-neoplastic cervical tissue lesions (erosion).
3. A stronger expression was observed for hMLH1 than for thMSH2 proteins - contrary to the cases of carcinoma of the uterine corpus and endometrial carcinoma.

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