Prognostic importance of selected molecular immunohistochemical markers and DNA ploidy in endometrial cancer

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Summary

The aim of the study was the analysis of the new molecular genetic immunomarkers (p53, c-erbB-2, Ki 67, bcl-2) hormonal receptors (ER, PR) and ploidy disturbances and their relation to the most important prognostic factors for endometrial cancer. The study group consisted of 135 endometrial cancer patients. Biopsies of the tumours obtained at operations were routinely histopathologically examined. Subsequenly, the immunohistochemical tumour markers were determined. The same biopsies were examined by microdissection and flow cytometric ploidy analysis and karyotyping. The findings were compared with the most important prognostic factors for endometrial cancer, mainly with clinical stage of the disease and grade. *Results:* High expression of p53, Ki 67, c-erbB-2 and low rate of progesterone receptors was found in the prognostically unfavourable group (G 3). Aneuploidy was found in 72% in the group of poorly differentiated endometrial cancers (G 3) in contrast to 27% in the group of G1 and G2 tumours, but this difference was not statistically significant. *Conclusions:* Identification of p53, Ki 67, c-erbB-2, PR and determination of DNA ploidy is a useful tool to specify a group of prognostically unfavourable patients.

Key words: Endometrial cancer; DNA ploidy; Molecular immunomarkers; Prognostic factors.

Introduction

Endometrial carcinoma is the second most frequent gynaecological malignancy (after breast carcinoma) affecting female reproductive organs. Continuing increase of incidence, mainly in economically developed countries, is evidently related to the complex influence of civilization factors such as prolonged survival, reproductive behaviour and living standards of contemporary populations. In spite of the relatively low mortality, almost as many women in the Czech Republic die from endometrial carcinoma as those from cervical carcinoma due to the lower incidence of the latter.

Adequate, complex and timely initiated treatment is essential for good therapeutic results. Adequate therapy of malignant tumours in general must bring about the maximal therapeutic effect with the minimal burden for the patient. Undertreatment as well as overtreatment should be avoided.

To select the right therapeutic strategy it is necessary to take into account a whole array of factors among which prognostic factors are very important. Even though the clinical and pathological parameters such as grade and histopathologic type of the tumour are still the most important factors, there are several other factors which can influence and predict the course of the disease and are, therefore, important for the estimation of the extent of surgery and eventually the necessity of following radiotherapy or other adjuvant therapies. Molecular immunomarkers and DNA ploidy of malignant cells are also among those important prognostic factors [1, 2].

Research in the field of molecular genetic biomarkers is at present rapidly growing in oncology and in oncogynaecology as well. New biomarkers are important not only for the theory where they contribute to answering the basic questions concerning the course of cancerogenesis, but they also have an impact in clinical medicine [3].

It is already known that the malignant process is initiated by changes in genetic information in normal cells. The main event in the initial phase of carcinogenesis is the activation of prooncogenes, inactivation of tumour suppressor genes, microsatelite instability and several other genetically related changes. The chromosomal genome often undergoes important changes which occur either in an individual nucleotide or also, in the whole chromosome. This often leads to the loss of heterozygosity, aneuploidy or in some cases also polyploidy. The extent and character of these defects is thereafter directly reflected in the biological behaviour of the tumour and in this way determines the clinical course of the disease.

The aim of this study was to evaluate selected immunomarkers (p53, c-erB-2, Ki 67, bcl-2), hormonal receptors (ER, PR) and changes in cell ploidy of malignant cells and compare them with the most important prognostic factors, mainly with the clinical stage and grade and eventually histopathological type of the tumour [4].

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Material and Methods

The study included 135 patients with histopathologically proven endometrial carcinoma. The diagnosis was based on the examination of bioptic material obtained by diagnostic hysteroscopy or D&C. The patients underwent surgery which in most cases included hysterectomy with bilateral salpingo-oophorectomy. If indicated pelvic and paraaortal lymphadenectomy were performed. The surgery was done from the abdominal, vaginal or laparoscopic approach.

Indirect immunohistochemistry on formalin-fixed paraffin-embeded 5-8 μm thick tissue sections was performed in the staining automaton Vetana Benchmark, using mouse or rabbit mouse primary monoclonal antibodies against p53, c-erB-2, Ki 67, bcl-2, PR and ER. For negative controls, the samples were taken through the procedure with omission of primary antibody. Positive staining was expressed in percent. The median value was set as a limit of positivity for p53, c-erB-2, Ki 67 and bcl-2. The cut-off limit for p53 and bcl-2 was 20%, for Ki-67 40%, and 10% for c-erbB-2. As for the PR and ER the lower limit of positivity was 5%.

DNA ploidy was also examined from the samples of microdisected paraffinized tumorous tissue using flow-cytometry. The stained nuclei were measured by the flow-cytometer FACS Calibur and the obtained data were analysed by ModFit-LT software. DNA histograms were classified as diploid or aneuploid with respect to the content of DNA in the region of the Go/Gl peak. The findings were compared with the internal standard of nuclei isolated from normal diploid cells. If the quality of the sample enabled further measurements, the analysed nuclei were also evaluated for the cell cycle proliferation.

The study group was divided into two groups according to surgical staging. The first group with a good prognosis comprised 58 patients with FIGO classification IA and IB. The group with a poor prognosis included 77 patients with advanced disease, classified as FIGO IB, II, III and IV.

According to the grade of tumour the patients were divided into 113 with a good prognosis (FIGO G1 and G2) and 22 with a poor prognosis (G3).

The main histopathological classification of the tumour, endometroid cancer, was found in 130 patients. The remaining five patients had serous papillary adenocarcinoma or mixed types of tumours. With regard to the relatively small number of prognostically unfavourable cases, these were not evaluated separately.

According to karyotype, the tumours were divided in euploid or aneuploid groups. The aneuploid group included hypoploid and polyploid nuclei.

The results of immunomarkers, estrogen and progesterone receptor analysis as well as cell ploidy were evaluated in relation to the expected prognosis of the disease. Inclusion criteria for a good or bad prognosis were based either on FIGO staging or the grade of tumour.

Statistical evaluation was performed by arranging the data into contingency tables (cross tabulation) and calculating Fisher's tests for homogeneity of the compared groups of patients. The value of $p = 0.05$ was taken as significant in Fisher's tests. The statistical software used was SSPS Inc Chicago, USA. The results with both-sided exact significance less than 0.05 were considered statistically significant.

Results

Table 1 shows the division of the groups according to the grade of tumour.

Table 1. — *Tumour grades.*

G1-3			135	
	G1-2		113	83.7%
		euploid	48	42.5%
			p53 15	31.3%
			bcl-2 21	43.8%
			c-erbB-2 7	14.6%
			Ki-67 8	16.7%
			ER 42	87.5%
			PR 44	91.7%
		aneuploid	65	57.5%
			p53 19	29.2%
			bcl-2 31	47.7%
			c-erbB-2 6	9.2%
			Ki-67 20	30.8%
			ER 53	81.5%
			PR 59	90.8%
	G3		22	16.3%
		euploid	6	27.3%
			p53 5	83.3%
			bcl-2 4	66.7%
			c-erbB-2 1	16.7%
			Ki-67 3	50.0%
			ER 6	100.0%
			PR 4	66.7%
		aneuploid	16	72.7%
			p53 8	50.0%
			bcl-2 6	37.5%
			c-erbB-2 5	31.3%
			Ki-67 11	68.8%
			ER 13	81.3%
			PR 9	56.3%

In the group of 113 patients with the prognostically favourable grade (G1 and G2) there were 48 tumours (42%) with euploid karyotype, while in 65 tumours (57%) there were hypo- or polyploid nuclei. The respective expression of the studied immunomarkers and steroid receptors in euploid and aneuploid tumours is given in Table 1.

Among 22 patients with biologically immature tumours (G3) the euploid karyotype was found in six (27%) while aneuploid karyotype was found in 16 cases (72%). Though the difference seems to be high, it was not statistically significant.

Expression of p53 in the group with the prognostically favourable grade (G1 and G2) occurred in 34 patients (29%) while in the group G3 it occurred in 13 (59%). The difference was statistically significant. The same was valid also for the expression of Ki 67 (28 vs 64%) and c-erbB-2 (11 vs 27%). Expression of bcl-2 was not significantly statistically different.

Expression of steroid receptors differed significantly only in case of progesterone receptors. The well differentiated tumours were positively stained in 100%, while the less differentiated ones only in 59% (Table 2).

Clinical stage FIGO IA + IB was found in 58 (43%) patients. Euploid cells were found in 20 patients (34%)

Table 2. — *Figo stages.*

Figo I-IV			135	
	Figo IA+IB		58	43.0%
		euploid	20	34.5%
			p53 7	35.0%
			bcl-2 10	50.0%
			c-erbB-2 5	25.0%
			Ki-67 3	15.0%
			ER 16	80.0%
			PR 20	100.0%
		aneuploid	38	65.5%
			p53 10	26.3%
			bcl-2 17	44.7%
			c-erbB-2 3	7.9%
			Ki-67 12	31.6%
			ER 30	78.9%
			PR 34	89.5%
	Figo IC-IV		77	57.0%
		euploid	34	44.2%
			p53 13	38.2%
			bcl-2 15	44.1%
			c-erbB-2 3	8.8%
			Ki-67 8	23.5%
			ER 32	94.1%
			PR 28	82.4%
		aneuploid	43	55.8%
			p53 17	39.5%
			bcl-2 20	46.5%
			c-erbB-2 8	18.6%
			Ki-67 19	44.2%
			ER 35	81.4%
			PR 34	79.1%

and aneuploid karyotype was found in 38 (66%) cases. Expression of studied immunomarkers and steroid receptor positivity in the same group of euploid and aneuploid tumours is given in Table 2.

Prognostically unfavourable stages FIGO IC, II, III, IV were found in 77 (57%) patients. There were 34 (44%) euploid tumors and 43 (56%) aneuploid tumours. The difference was not statistically significant.

Comparison of studied immunomarkers between prognostically favourable stage FIGO IA, IB and stages FIGO IC, II, III, and IV did not show statistically significant differences. On the contrary, statistically significant differences were found for progesterone receptors, which were more frequently positive in the initial, prognostically favourable disease.

Discussion

At present it is generally accepted that the genesis of malignant tumour is determined by a primary genetic disorder, either sporadically arising or based on hereditary disposition. Cancerogenesis is a multistep process, originating as imbalance between normal cellular proliferation and apoptosis, *i.e.* programmed cell death. Many genes can participate in the formation of tumours. The main events in the initial phase of cancerogenesis are changes in activation prooncogenes, inactivation of tumour suppressor genes, microsatellite instability, aneuploidy, point mutations, translocations, amplification, loss of heterozygosity and others.

Oncogenes which arise by a defect – mutation – of prooncogenes are pathologic. They encode proteins which, if they occur in an abnormal amount or form, can lead to the tumorous transformation of cells. Genes as c-erbB-2, also known as HER2/neu and bcl-2, belong to the well known oncogenes [5]. Oncogenes can also form complexes with the products of tumour suppressor genes which leads to their inactivation.

Amplification with increased expression of c-erbB-2 was reported in 10-40% of endometrial cancers [6, 7]. Oncogene c-erbB-2 encodes in a similar way as does EGRF (epidermal growth factor receptor) and is therefore frequently found in aggressive types of tumours and should correlate with a worse prognosis. Nevertheless, the conclusions of clinical studies are ambiguous. Our study shows an increased expression of this marker in prognostically unfavourable cases according to the grade, but not according to FIGO stage.

Oncoprotein bcl-2 inhibits apoptosis and prolongs the cellular lifespan. Its expression changes in the course of the menstrual cycle; it is high in the proliferative phase and low during menstruation. High levels are also found in endometrial hyperplasia. Increased expression has been documented in endometrial cancer, especially in carcinoma of the type I. It is suggested to be related to the greater depth of myometral invasion, stage, grade and a worse prognosis [8-10]. Decreased expression is reported in type II carcinoma and namely in unfavourable histopathological types of tumour [10, 11]. We found increased levels of bcl-2 in prognostically unfavourable cases according to the grade, but not according to the stage. The results, however, did not reach statistical validity.

Tumour suppressor genes play an important role in cell division. They encode proteins which inhibit/regulate cell division. They can control cell division in two ways - either they rectify the arising errors or they stop further division of cells. They function as the so called "safety catch" which switchs off the cell cycle in case of abnormal proliferation or a defect of genetic information. The defect of tumour supressor genes such as mutation can result in the escape of defective cells from their control mechanisms and in this way contribute to the formation and growth of malignant tumours.The most known suppressor gene is p53. With regard to its important role in the process of apoptosis it is also called the "genome guardian". It encodes a nuclear phosphoprotein and as a transcription factor influences expression of other genes which regulate growth and division of cells. Gene p53 relatively often undergoes mutation and its specific regulatory role may change or is inhibited. Protein product of the mutated gene is slowly degraded in malignant cells; its regulation does not respond to the dynamic changes in cells and therefore it can be detected by immunohistochemical methods. In endometrial cancer, p53 is detected

very often and its increased expression correlates with the clinical stage of disease, poor prognosis and aggressive histopathological types of tumour [6, 7, 11-14]. Our results show almost three times higher values in poorly differentiated tumours compared to well differentiated ones. The difference was highly statistically significant ($p = 0.01$). The difference in expression of p53 according to the stage was not statistically significant.

Marker Ki-67 is connected with cell proliferation which is a characteristic feature of all malignant tumours. This protein is encoded by the MK 167 gene and can be detected in cell nuclei in all phases of the cell cycle except for the quiescent phase (G0). The majority of endometrial cancers express a low proliferative index, while increased expression usually correlates with unfavourable grade, clinical stage and means a poor prognosis. Unfavourable histopathological types of endometrial cancer also show high expression [15-17]. Our study documented increased expression in prognostically unfavourable tumours according to grade. High positivity was found mainly in uneuploid and immature tumours (69%). The results were statistically significant. We could not evaluate the importance of Ki-67 in non-endometroid cancers due to the small number of patients.

Estrogen and progesterone receptors, when activated, bind to the specific target sites in DNA where they modulate expression of respective genes. Besides the direct activation of target genes, the indirect mechanism of effect through the binding on transcription factors has also been reported [18]. Steroid receptors play an important role not only in the healthy endometrium, but participate also in the process of endometrial carcinogenesis. The absence of steroid receptors is considered a negative prognostic factor and is most often found in case of unfavourable histopathologic types and aggressive tumours of the endometrium [9, 18-20]. In agreement with published data our study showed a statistically significant lower occurrence of progesterone receptors in biologically immature tumours as well as in advanced stages of tumour (FIGO IC-IV).

A typical karyotype of healthy human cells consists of 46 chromosomes assigned in 23 pairs. In contrast, genetic material in tumour cells is characterised by a certain degree of genetic instability. As a consequence of mitotic defects this may lead to important changes of a genome. The marked elements of tumour progression are defects in genes which are connected with the maintenance of chromosome stability and integrity. The excess or loss of one or more chromosomes leads to genetic instability in tumour cells. It has been documented that this instability is an early manifestation of malignant transformation and that it is typical mainly for some types of malignant tumours [21]. Aneuploid changes are reported in a wide range (15-40%) in endometrial carcinoma [22-27]. In general, the well differentiated prognostically favourable tumours show the prevalence of diploid cells, while the aggressive tumours have typically aneuploid karyotype [29-32]. From the clinical point of view it is important that most authors consider ploidy as an independent prognostic factor [38-42]. In the prognostically borderline cases (FIGO IC, G1-2) the determination of ploidy can contribute to the decision about adjuvant therapy.

In our group of patients the aneuploid forms of karyotype were more frequent in biologically immature tumours (G3). Though, the difference between prognostically favourable stages G1 and G2 and unfavourable grade G3 was not statistically significant.

Furthermore a higher proportion of aneuploidy in tumours of stages FIGO IC-IV was not found.

Conclusion

The determination of DNA ploidy, steroid receptors and selected immunohistochemical markers, mainly p53, Ki 67 and c-erbB-2 should contribute to specify the groups of endometrial carcinoma with favourable and unfavourable prognoses. This should lead to the selection of an optimal therapeutic strategy. However the DNA ploidy did not prove to be a significant prognostic factor in our study.

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