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IDENTIFICATION OF NEW DNA MARKERS OF ENDOMETRIAL CANCER IN PATIENTS FROM THE UKRAINIAN POPULATION

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Aim: **To identify clinically significant molecular markers of endometrial cancer.** ***Materials and Methods:*** **Cancer and normal endometrial tissue samples from 20 patients of the Gynecology Clinic of Odessa State Medical University (Odessa, Ukraine) with confirmed endometrial cancer were compared for SSR and ISSR polymorphisms. Identified polymorphic fragments from anonymous genome regions situated between microsatellite repeats underwent direct DNA sequencing; analysis of their homology to sequences from human genome database has been performed.** ***Results:*** **No significant variability for the microsatellite loci adjacent to the *E2F1*, *BAX*, *TCF7L2*, *C-MYC*, *WNT1*, *FES*, *DCC*, *P27*, *THRA*, *APC*, *CYP19* and *P53* genes was detected. Search for new molecular markers of endometrial cancer within anonymous DNA sequences located between microsatellite repeats revealed 100 bp and 174 bp polymorphic fragments. These fragments were detected correspondingly in 60% and 35% of patients. 100 bp fragment appeared to be homologous to a region within the *NFKB* gene, 174 bp fragment – to a sequence within the *DDR1* gene.** ***Conclusions:*** ***NFKB1* and *DDR1* genes may be regarded as potential markers for some types of endometrial cancer. This is a first report about possible association of these genes with endometrial cancer.**
Key Words: **SSR-marker, ISSR-marker, endometrial cancer, sequencing.**

Endometrial cancer is recognized worldwide as one of the most common oncological disorders of the female genital tract. At present this disease is being diagnosed in 70% of cases at stage III–IV, when it is already quite difficult to find an effective treatment. Molecular changes in structure of several genes have recently been reported to lead to endometrial cancer progression. Up to 83% of hormone-dependent endometrioid carcinomas are associated with the loss-of-function mutation in the tumor suppressor *PTEN* gene located on chromosome 10. Altered *PTEN* expression is considered as a marker of the earliest endometrial precancers and good survival prognosis, while methylation of its promoter is associated with late-stage disease [1, 2]. Some data suggest that structural alterations in p53, p16, K-ras, as well as Her2/neu overexpression may have prognostic value, though without relation to histological type and stage [3–8]. Predictive significance of K-ras mutations may depend on age at disease onset. However, contradictory reports and polygenic nature of the disease do not allow to consider the known molecular markers quite reliable. Thus, further detailed investigation of genetic components related to endometrial cancer progression is needed to identify additional molecular markers of clinical significance.

For this purpose we conducted our study in two directions: 1. Detection of molecular polymorphisms in microsatellite loci adjacent to the known *E2F1*, *BAX*, *TCF7L2*, *C-MYC*, *WNT1*, *FES*, *DCC*, *P27*, *THRA*, *APC*, *CYP19*, *P53* genes and estimation of their association with endometrial cancer risk. 2. Search for the potential molecular markers in anonymous DNA sequences located between microsatellite repeats (inter simple sequence repeat polymorphism) in human genome, sequence analysis of polymorphic fragments, their identification in human genome database and evaluation of their possible role in endometrial cancer progression.

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Abbreviations used: APC – adenomatosis polyposis coli; *BAX* – BCL2-associated X protein; *CYP19* – cytochrome P450 family 19 subfamily A polypeptide 1; *DCC* – deleted in colorectal carcinoma; *DDR1* – discoidin domain receptor family member 1; *E2F1* – E2F transcription factor 1; *FES* – feline sarcoma oncogene; ISSR – inter-simple sequence repeats; *NFKB1* – nuclear factor of kappa light polypeptide gene enhancer in B-cells 1; *PTEN* – phosphatase and tensin homolog; SSR – simple sequence repeats; *TCF7L2* – transcription factor 7-like 2; *THRA* – thyroid hormone receptor alpha; *WNT1* – wingless-type MMTV integration site family member 1.

MATERIALS AND METHODS

The study cohort was comprised of twenty women with surgically and histologically confirmed endometrial cancer who underwent surgery at the Gynecology Clinic of Odessa State Medical University, Odessa, Ukraine. All subjects were informed and gave written consent to participate in the study and to allow their biological samples to be genetically analyzed. Approval for this study was obtained from the Scientific Council of Odessa State Medical University.

DNA was extracted from the tumor and normal endometrial tissue samples of the same patients using "DNA purification kit" (Promega Corporation, USA) according to the manufacturer's recommendations. DNA was stored at 4 °C until analyzed.

The amplification of 12 microsatellite loci adjacent to the known *E2F1, BAX, TCF7L2, C-MYC, WNT1, FES, DCC, P27, THRA, APC, CYP19, P53* genes was performed. The primers and PCR conditions are summarized in Table 1.

Four ISSR primers were used to discover new potential DNA markers: ISSR1 — (GTG)7A, ISSR12 — (TG)9C, ISSR14 — (AC)9G, ISSR17 — (CA)10G. The primers were synthesized by the MWG-BIOTECH (Germany). PCR mix in a volume of 25 μl contained 50 mM KCl, 20 mM Tris-HCl (pH 8.4 at 25 °C), $MgCl_2$ (2 mM for ISSR-primers and 4 mM for SSR-primers), 0.01% Tween-20, 0.15 mM each dNTP, 0.2 μM primers, 10-20 ng DNA, 0.8-1 U Taq-polimerase.

Table 1. The primer sets and PCR conditions

Primer	Sequence	Annealing (°C, s)
E2F1-f	5′-TGCAGAAGTGGCCTTAGCAA-3′	58, 30
E2F1-r	5′-ATCATTGAACGAACAGGGGG-3′	
BAX-f	5′-GCTCACTTTCACTGAGGATGC-3′	50, 30
BAX-r	5′-TTAGGCCTAGCAGAGAATCACC-3′	
TCF7L2-f	5′-AGTGTGACTCTGGCCAAGCT-3′	60, 30
TCF7L2-r	5′-TGCTCTTTAAGGCACTCTTGC-3′	
C-MYC-f	5′-CGTTAGAAAGGTCTCTGGAC-3′	56, 30
C-MYC-r	5′-GTCTTAGGTAAGAATTGGCA-3′	
WNT1-f	5′-AGCTCTCACACACTCTCCTTCC-3′	58, 30
WNT1-r	5′-GGAAAGTTAAAGAGGCATCCG-3′	
FES-f	5′-GCTTGTTAATTCATGTAGGGAAGGC-3′	55, 30
FES-r	5′-GTAGTCCCAGTCACTTGGCTACTC-3′	
DCC-f	5′-GATGACATTTTCCCTCTAGA-3′	56, 30
DCC-r	5′-TTTAGTGGTTATTGCCTTGAA-3′	
P27-f	5′-GGCACTTCCCAGCATGTAGCCG-3′	60, 30
P27-r	5′-GTGGCCACATGGAGTGACCTGGGCCTC-3′	
THRA-f	5′-CTGCGCTTTGCACTATTGGG-3′	60, 30
THRA-r	5′-CGGGCAGCGTAGCATTGCCT-3′	
APC-f	5′-AGCAGATAAGACAGTATTGCTAGTT-3′	50, 30
APC-r	5′-ACTCACTCTAGTGATAAATCGGG-3′	
CYP19-f	5′-ACAGGCAAGTGGCTGAGG -3′	58, 30
CYP19-r	5′-ATTCAGCATTGACCCTTGC-3′	
P53-f	5′-AGGGATACTATTCAGCCCGAGGTG-3′	58, 30
P53-r	5′-ACTGCCACTCCTTGCCCCATTC-3′	

After the amplification the SSR-PCR products were analyzed by gel electrophoresis on 10% polyacrilamide gels under denaturation conditions, the ISSR-PCR products — on 6% plolyacrilamide gels. Molecular weight of the PCR products was calculated with "Onedscan" software using pUC18/MspI as a standard.

Polymorphic DNA fragments were eluted from gel and reamplified with M13-tailed primer 5′-TGT AAA ACG ACG GGC CAG T-ISSR17–$(CA)_{10}$G to generate products for sequencing. PCR was carried out in a total volume of 10 μl containing 20–50 ng of genomic DNA, 4–6 pmol primer, 200 μM dNTPs, 50 mM KCl, 2.5 mM $MgCl_2$; 10 mM Tris-HCl (pH 8.3), 1.5% DMSO, 50 mM TMAC, and 0.2 U Tfi-polymerase. Quality of the PCR-products was analysed in 1% agarose gel. The intensive major bands were cut out of the gel, purified with a GFXTM PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech) and applied to direct DNA sequencing. The sequencing reaction was performed with M13 primer and a ThermoSequenase 7-deaza-dGTP Sequencing Kit (Amersham Pharmacia Biotech) using the BioRad ICycler. The process included 20 cycles of 30 s at 95 °C, 30 s at 54 °C and 1 min at 72 °C. The products were resolved on 25 cm 7% denaturing Long Ranger gels run on a Li-Cor 4000 sequencer, following the loading protocols. Sequences were base-called using the Base ImagIR™ 4.0 (LI-COR) software. Sequence homology was determined using the NCBI Blast Software (http://www.ncbi.nlm.nih.gov).

RESULTS

DNA fragments of 12 microsatellite loci were amplified with primers and under conditions listed in Table 1. No polymorphism was detected for 10 of them at comparison of different genotypes, or different samples from the same patient (tumor versus normal tissue). Genotypic differences were discovered for the *DCC* (170–170, 170–200, 195–195, 195–200 bp genotypic variants) and *FES* (240–240, 240–244, 244–244 bp genotypic variants) loci. However, the fragments from different samples of the same genotypes did not differ in their pattern with the only exception: in one genotype a 200 bp fragment was absent in normal tissue, but present in cancer sample.

In this study we also performed amplification of DNA fragments with 4 ISSR-primers. The ISSR17–$(CA)_{10}$G generated 100 bp and 174 bp polymorphic fragments (Figure). Polymorphic 100 bp DNA fragment was detected in twelve patients (60%). In two of them it was absent in cancer samples, in the rest of the patients it was present both in cancer and normal tissue samples. Polymorphic fragment of 174 bp in length was detected in seven patients (35%). In four of them it was identified only in normal tissue samples, in one patient – in both sample types, and in two patients – only in cancer samples. We performed sequencing of both fragments and checked their homology to the known genes using human genome database. The analysis included only sequences with known functions; cloned sequences or sequences with supposed functions were ignored. Sequences homologous to 100 bp and 174 bp fragments were detected, correspondingly, within the *NFKB1* and *DDR1* genes (Table 2).

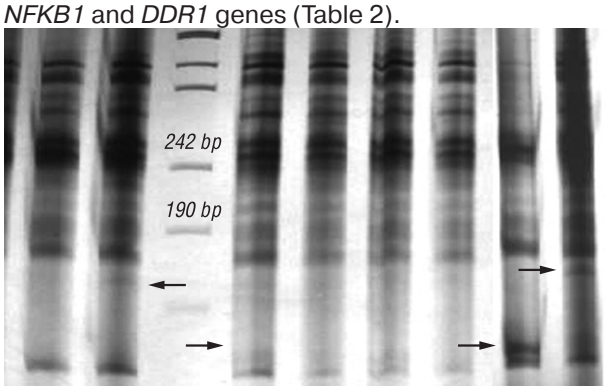

Figure. Products of DNA amplification by ISSR17 (CA) 10G primer. C — tumor sample, N — normal tissue sample, M — molecular weight marker pUC18/MspI. 100 bp and 174 bp polymorphic DNA fragments are indicated by arrows

DISCUSSION

Microsatellites are short, simple repeated sequences of DNA distributed throughout the human genome. The accumulation of insertion or deletion mutations in these repetitive sequences results in a form of genomic instability, called microsatellite instability (MSI). The MSI has been reported in a variety of both hereditary and sporadic tumors, including endometrial cancer, and has been shown to have prognostic significance [9]. However, in the present study we failed to detect significant variability for microsatellite loci adjacent to the *E2F1*, *BAX*, *TCF7L2*, *C-MYC*, *WNT1*, *P27*, *THRA*, *APC*, *CYP19*, *P53* genes in cancer and normal tissue samples obtained from the endometrial cancer patients. Genotypic differences were detected only for the loci located next to the *DCC* and *FES* genes, but fragments from normal and cancer tissue samples of the same genotypes did not differ in their pattern. Of note, *DCC* regulates normal endometrial cell growth

and is recognized as the tumor suppressor gene for endometrial cancer [10].

In our previous study [11] we compared the variability of the above-mentioned loci in patients with leiomyoma, a benign neoplasm, using the normal myometrium as a control tissue. According to our observations, detected polymorphism reached 40% at the genotypic level, and up to 60% in neoplastic and normal tissue within the same genotype. The SSR-markers are far less informative in the present study, which may be explained by differences in genetic pathways involved in leiomyoma and endometrial cancer progression. On the basis of our data, we conclude that the analyzed microsatellite loci adjacent to the *DCC* and *FES* genes cannot be regarded as markers of endometrial cancer.

Search for the potential markers for endometrial cancer in anonymous DNA sequences located between microsatellite repeats revealed 100 bp and 174 bp fragments. The first fragment detected in 60% of patients appeared to be homologous to a sequence within the *NFKB1* gene. *NFKB1* is one of the genes controlling immune response; loss of its expression suppresses the immune system and apoptosis, disrupts the regulation of the genes involved in cell-to-cell interaction, intercellular communication and spreading of primary pathogenic signals [12]. Thus, mutation just in one *NFKB1* gene may trigger a set of pathological processes leading to tumor formation. The discovered polymorphic fragment was lost in two cancer samples, which means that such a loss may affect gene function. Although in ten patients this fragment was detected both in cancer and normal tissue sample, it has to be mentioned that we did not perform microdissection in the present study, so DNA derived from tumor samples could in fact contain some DNA from non-malignant cells. The presence of this fragment both in tumor and normal tissue samples of some patients may partly be explained by such DNA heterogenity.

Based on the role of *NFKB1* in tumorigenesis [12] and our own observation, we conclude that the *NFKB1* gene may be regarded as a potential informative marker for endometrial cancer progression. However, a more detailed study of its role in tumorigenesis must be performed in a bigger population of cancer patients with the use of microdissection.

Another polymorphic fragment of 174 bp in length, detected in 35% of patients, appeared to be homologous to a sequence within the *DDR1* gene. It is known that *DDR1* is overexpessed in some human tumors, e. g. breast, ovarian, esophageal and brain tumors [13–15]. As far as we know, no data concerning its involvement in endometrial cancer progression have been published previously. However, evidence for its activity in epithelial cells indirectly confirms such suggestion. Besides, it plays a key role in regulation of cellular growth, differentiation, metabolism, intercellular communications and interaction of cells with their environment. Suggestion about relation of functional alterations in such a key gene to endometrial cancer progression needs to be confirmed in a more detailed study. The results presented here show that the polymorphic fragment may be present not only in cancer samples, but also in normal tissue. Absence of regularities in the populational distribution of these fragments restricts to some extent the possibility of the wide application of such markers. On the other hand, polymorphism within the same genotype reflects the specific ways of cancer progression. Investigation of such ways will tailor modern clinical medicine to each patient according to his/her genetic fingerprints and drive state-of-the-art screening and prevention of different cancer forms to a new level. On the basis of our observations we suggest that *DDR1* may influence to some extent endometrial cancer development, though its effect may depend on genotype and/or stage of the disease.

In conclusion, polymorphic 100 bp and 174 bp DNA fragments, which may be regarded as potential markers of endometrial cancer, were detected in our study. DNA sequence analysis revealed their homology to sequences within the *NFKB1* and *DDR1* genes. This a first report on possible association of *NFKB1* and *DDR1* with endometrial cancer. The pattern of populational distribution of discovered polymorphic fragments indicates that *NFKB1* and *DDR1* may be involved in some specific pathways of endometrial cancer development, but their likely role in pathological processes demands further investigation in a more detailed study using microdissection.

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Table 2. Identified polymorphic DNA fragments and functions of corresponding genes (from Human Genome Database)

Polymorphic fragment 100 b.p.	gggaggcagaggttgcagtgagccgagatcataccattgcactccag cctgggcaacagcagcgaaactccgtctcaaaaaataaataaaatatctgtgtgt-gtgtgtgtgtgtg (primer)	
Gene	Locus	Function
Homo sapiens nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (*NFKB1*)	NT_011786	Regulation of the genes involved in cell-to-cell interaction, intercellular communication, cell recruitment or transmigration, amplification or spreading of primary pathogenic signals, and initiation or acceleration of tumorigenesis. Regulation of the immune system and programmed cell death [12]
Polymorfic fragment 174 b.p.	ccagatggactcctgtcttacaccgcccctgtggggcagacaatgtatttatctgaggccgtgtacctcaacgactccacctatgacggacataccgtgggcggg-taagaaaggcccctgcaggatatggagtttggggtgggagggaggactctgtgtgtgtgtgtgtgtg (primer)	
Homo sapiens discoidin domain receptor family, member 1 (*DDR1*)	NM_013993	Plays a key role in the communication of cells with their microenvironment. Regulation of cell growth, differentiation and metabolism. Plays a role in cell-to-cell contact and in cell adhesion signaling pathways [13–15]

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ИДЕНТИФИКАЦИЯ НОВЫХ МАРКЕРОВ РАКА ЭНДОМЕТРИЯ В ГРУППЕ ПАЦИЕНТОВ ИЗ УКРАИНСКОЙ ПОПУЛЯЦИИ

Цель: **идентификация клинически значимых маркеров рака эндометрия.** ***Материалы и Методы:*** **проведен анализ SSR- и ISSR-полиморфизма в образцах опухолей и непораженной ткани эндометрия двадцати пациентов, получавших лечение в клинике гинекологии Одесского государственного медицинского университета (Одесса, Украина). Выполнено секвенирование выявленных полиморфных фрагментов ДНК, локализованных в анонимных участках генома между микросателлитными повторами (ISSR-полиморфизм), осуществлен анализ их гомологии с известными участками ДНК из базы данных генома человека.** ***Результаты:*** **не установлено значительной вариабельности микросателлитных повторов, соседствующих с генами *E2F1, BAX, TCF7L2, C-MYC, WNT1, FES, DCC, P27, THRA, APC, CYP19* и *P53*. В процессе поиска новых маркеров рака эндометрия среди анонимных последовательностей ДНК, локализующихся между микросателлитными повторами, выявлены полиморфные фрагменты длиной 100 и 174 пн. Эти фрагменты присутствовали соответственно у 60 и 35% пациентов . Фрагмент длиной 100 пн оказался гомологичным участку гена *NFKB1*, а фрагмент длиной 174 пн – участку гена *DDR1*. *Выводы:*** **гены *NFKB1* и *DDR1* могут рассматриваться в качестве потенциальных маркеров некоторых типов рака эндометрия. Это первое сообщение о возможной ассоциации данных генов с опухолями эндометрия.**

Ключевые слова: **SSR-маркер, ISSR-маркер, рак эндометрия, секвенирование.**

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