Characterization of genomic changes in the cervical pre-cancerous lesions and tumors induced by different types of human papillomaviruses

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Abstract Cervical carcinoma is the second most common malignancy among women in both incidence and mortality. Although much is known about the etiology and treatment of cervical cancer, the role of genetic alterations in the multistep pathway of cervical tumorigenesis is largely unknown. The aim of this study was to characterize the genomic changes in the cervical pre-cancerous lesions and tumors, induced by different types of human papillomaviruses. In this research was used the BlueGnome CytoChip oligo 2 × 105 K microarray for whole-genome oligo-array CGH. Microarray CGH analysis of 40 specimens was carried out—12 specimens from patients with early-stage squamous cell carcinomas; 19 specimens from patients with mild to moderate dysplasia and 9 with severe dysplasia. First we performed microarray CGH analysis of five DNA pools which contained the DNA from homogeneous groups of patients. The results revealed presence of micro chromosomal aberrations in chromosome region 14q11.2. According to the genome database these aberrations represent polymorphisms. Microarray analysis of DNA from 9 separate carcinoma lesions revealed a total of 26 aberrations in 14 chromosomes of nine patients. Our

results showed the advantages of high-resolution chips in the clinical diagnosis of patients with cancerous and pre-cancerous lesions caused by viral infection with HPV, but also highlight the need for extensive population studies revealing the molecular nature and clinical significance of different CNVs and the creation of detailed maps of variations in the Bulgarian population. This would facilitate extremely precise interpretation of specific genomic imbalances in the clinical aspect.

Keywords Human papillomavirus · Microarray CGH analysis · Genomic aberration · CNV · Cervical cancer

Introduction

Cervical cancer (CC) is the second most common cancer in women worldwide, affecting 500,000 individuals each year, and it is the main cause of death of women with cancer in developing countries. Despite the damage caused by the oncoviral proteins, CC is a rare complication of the viral infection because most infections are transient and do not evolve into neoplastic lesions. Although 95 % of the patients with precancerous lesions harbor HPV, only a small fraction of the cases eventually progress to invasive cancer [25]. On average, it takes 12–15 years before a persistent HPV infection may, via the premalignant stages of cervical intraepithelial neoplastic lesions (CIN), lead to CC. These findings suggest HPV infection alone does not cause the disease and other factors, such as abnormal host genes, could be associated with the development of invasive cancer. Further identification of such genetic alterations is critical in our understanding of the molecular basis of CC development [18].

Several genomic regions have been identified with changes in the number of DNA copies copy number-altered

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regions (CNAs) in CC through the analysis of the tumor genome by using methods such as comparative genomic hybridization (CGH), fluorescence in situ hybridization (FISH), and microarrays of single nucleotide polymorphisms (SNPs) and copy number variations (CNVs). To date, a number of CGH studies have identified chromosomal changes involving loss of 2q, 3p, 4p, 4q, 5q, 6q, 11q, 13q and 18q regions and gain of 1q, 3q, 5p and 8q at various stages of CC [7, 17, 22]. Genomic imbalances can contribute to deregulated expression of oncogenes and tumor suppressor genes in cancer cells, and the accumulation of such altered genes has been correlated with tumor progression [9, 11, 14, 16]. However, the contribution of these alterations to cervical carcinogenesis is still a matter of debate.

Here we report a microarray CGH analysis of specimens in varying degrees of development of the disease and we found one micro chromosomal aberration in the microarray analysis of DNA pools and 26 chromosomal aberrations in the individual DNA microarray study.

Materials and methods

Biological samples

For this study, cervical specimens were used in accordance with the ethical principles applied by the Sofia's Medical University, "Tokuda" Hospital, Sofia and National Institute of Oncology and Hematology, Sofia. All legislative requirements to protect human rights have been respected as well.

Cervical smears with different cytological diagnoses were collected from patients in the following three hospitals—University Hospital of Obstetrics and Gynecology Majchin Dom, Sofia; Medical Center Tokuda, Sofia, and National Institute of Oncology and Hematology, Sofia.

Microarray CGH analysis of 40 specimens was carried out—12 specimens from patients with early-stage squamous cell carcinomas; 19 specimens from patients with mild to moderate dysplasia and 9 with severe dysplasia. They were classified according to the Papanicolaou classification system by the Department of Pathological Anatomy. For this virological study, cervical smears were used in accordance with the ethical principles applied by the Sofia's Medical University, "Tokuda" Hospital, Sofia and National Institute of Oncology and Hematology, Sofia. All legislative requirements to protect human rights have been respected as well.

DNA pools were constructed by mixing DNAs from homogeneous groups of patients (DNA pool 1—patients with carcinoma lesions and presence of HPV16 infection; DNA pool 2—patients with cancer lesions and the presence

of other types of HPV infection; DNA pool 3—patients with cervical dysplasia and presence of HPV16 infection; DNA pool 4—patients with cervical dysplasia and the presence of another type of HPV infection; DNA pool 5—patients with moderate cervical dysplasia without HPV infection).

Microarray CGH analysis

Whole-genome oligo-array CGH was performed using the BlueGnome CytoChip oligo 2X105 K microarray, v1.1. This array contained ~105 000 oligonucleotide probes spaced at an average distance of 35 kb based on the NCBI build (36) of the human genome. Arrays were scanned at 532 nm (Cy3) and 635 nm (Cy5), using a GenePix 4100A two-color fluorescent laser scanner (Axon Instruments, Union City, CA, U.S.A.). The images were analyzed by BlueFuse Multi, version 2.2. (BlueGnome, Cambridge, UK). Test and sex-matched reference genomic DNA (500 ng) was labeled by random-priming, using BlueGnome Fluorescent dUTP Labeling System. Labeled sample and control DNA were combined for each hybridization area. Subsequently human Cot I DNA, 10X blocking agent and 2× hybridization buffer (Hi-RPM) were added to each reaction tube, to block repetitive sequence binding of target DNA on the arrays, and to prevent nonspecific binding of probe DNA to the targets. The hybridization mixtures were then denatured at 95 °C for 3 min, and subsequently incubated at 37 °C for 30 min. The hybridization process was carried out in hybridization chambers in microarray oven (Scigene 777) at 65 °C for 40 h. The slides were washed to remove the unhybridized labeled DNA using standard wash buffers (Oligo aCGH Wash Buffer 1 and 2, Agilent). Arrays were scanned at 532 nm (Cy3) and 635 nm (Cy5) using GenePix 4100A twocolor fluorescent laser scanner (Axon Instruments, Union City, CA, U.S.A.). The images were analyzed by BlueFuse Multi, version 2.2. (BlueGnome, Cambridge, UK). The computer program extracted the raw data and all signals reflecting the dosage of the distinct genomic region can be assigned to their specific location within the genome. In data processing log₂ ratios of Cy3 and Cy5 intensities were generated for all hybridized clones. If the amount of Cy3 and Cy5 fluorescent intensities are equal in one spot, this region of the patient's DNA is interpreted as being normal/balanced; if a threshold of increased ratio of Cy3 to Cy5 is detected, a duplication of the patient's DNA is suspected; if the threshold of the Cy3/Cy5 ratio is decreased, a deletion is expected. Normal copy numbers were considered in log₂ ratio between −0.3 and +0.3, values above +0.3 were evaluated as duplications and the ones under −0.3—as losses (deletions).

Results and discussion

Microarray analysis of DNA pools

CGH microarray analysis and molecular karyotyping are widely used in modern molecular biology. The increasing resolution of DNA—microarrays and optimization techniques allow detection with high accuracy, as a larger aberration, and a large number of small CNVs, whose clinical importance in some cases are unclear. The aim of our study was to try to find an existing relationship between genomic disorders arising in the course of carcinogenesis and the effect of human papillomavirus. In this research we did microarray CGH analysis of DNA pools followed by analysis of individual DNAs.

First we performed microarray CGH analysis (array CGH) of five DNA pools which contained the DNA from homogeneous groups of patients (Table 1), each of which differs in cytological/histological diagnosis and viral findings.

We found polymorphic variants in the number of copies of the 14q11.2 (deletions/duplications with different lengths of the polymorphic fragment) in specimens with cervical dysplasia and positive for HPV16 (Pool 3) and specimens with cervical dysplasia and negative for HPV (pool 5). According to the genome database these aberrations represent polymorphisms. Aberrations were not detected in DNA pools composed of carcinoma lesions (pool 1 and 2) and those with dysplasia, positive for another type of HPV (pool 4). We assume that this is due to the expected greater diversity and individual characteristics of genetic findings. Therefore we performed individual DNA microarray analysis.

Microarray analysis of individual DNAs

Second we performed individual microarray analysis to identify any pathogenical aberrations in samples from patients with early stage of CC (Table 2).

Microarray analysis of DNA from 9 separate carcinoma lesions revealed a total of 26 aberrations (25 microstructural and 1 large aberrations) in 14 chromosomes (chromosomes 1, 3, 4, 6, 7, 8, 10, 11, 12, 13, 16, 17, 19 and 22) of nine patients. The amount of detected genomic changes ranged from 664 bp to 73Mbp. Highest genomic instability (3.7 aberrations per patient) was observed in specimens infected with HPV6, 39, 52, 61, 62 and 84. The average number of aberrations in HPV 16 positive carcinoma lesions was 2 aberrations per patient. Abnormal genomic disorders were not detected in specimens positive for HPV 6.

In four out of seven analyzed cervical samples from Pool 1 DNA aberrations were found due to the individual microarray analysis. Microstructural abnormalities in 8p11.23 (such as deletions/duplications) were found in 3 patients. In two it was a matter for the same aberration, but was not detected in DNA microarray analysis of the pool. The result shows that the frequency of 30 % (two patients out of 7) is not sufficient for the visualization of the aberration in DNA pools. Aberrations in 8p11.23, 4q13.2 and 10q11.22 represent polymorphisms that are not pathogenical. Proven pathogenical aberrations were deletion in 11q22.1, deletion in 1p36.23 and lack of 3q [2, 6]. Deletion in 12q24.21 is currently with unproven significance.

We found microstructural changes with genomic polymorphic nature in DNA pool 2—duplications of 8p11.23 and 4q13.2 and deletion of 6p21.32.

Aberrations with unknown clinical significance or pathogenic nature were found in DNA pool 4: deletions in 4p16.2, 7p15.2, 13q22, 16q21.1, 17p11.2, 17p13.3, 17q21.31, 19p13.3 and 22q13.33; and duplications in 1p36.33 and 1q31.3. Some of them were probably pathogenic deletions at 4p16.2, 17p13.3 and 22q13.33 [1]; the rest did not appear in the database for benign polymorphic variants.

One of the pathogenic aberration was a deletion in the long arm of chromosome 11 (11q22.2-q22.3), with 4,616,714 base pairs in size and covering 24 gene (*CNTN5*, *TMEM133*, *PGR*, *TRPC6*, *ANGPTL5*, *KIAA1377*, *C11orf70*,

Table 1 Results from the microarray analysis of DNA pools

DNA pool	Chromosome	Type of aberration	Position in bp		Size (bp)	Band	
			Start	End		Start	End
Pool 1		No change					
Pool 2		No change					
Pool 3	14	Loss	21,688,726	22,034,733	346,008	14q11.2	14q11.2
	14	Gain	21,958,674	22,034,733	76,06	14q11.2	14q11.2
Pool 4							
Pool 5	14	loss	21,803,329	21,981,318	177,989	14q11.2	14q11.2

DNA pool: DNA from homogeneous groups of patients. Chromosome: indicates the chromosome number according to the human karyotype. Type of aberration: loss—deletion of chromosomal material; gain—amplification of chromosomal material. Position in bp (start and end): indicates the first and the last base pair of the CNVs in the genome according to database of genomic variants. Size (bp): the size of CNV in base pairs. Band (start and end): the cytlocus of the CNVs in the chromosome according to human ideograms

Table 2 Results from the individual microarray analysis

Patient	Chromosome	Type of aberration	Position in bp		Size (bp)	Band	
			Start	End		Start	End
Patient 1	4	Gain	69,057,765	69,165,843	108,079	4q13.2	4q13.2
	8	Gain	39,356,625	39,402,463	45,838	8p11.23	8p11.23
	11	Loss	99,082,481	103,699,195	4,616,714	11q22.1	11q22.3
Patient 2	4	Gain	69,057,765	69,165,843	108,079	4q13.2	4q13.2
	12	Loss	112,882,259	113,042,872	160,613	12q24.21	12q24.21
Patient 5	8	Gain	39,356,625	39,464,607	107,982	8p11.23	8p11.23
Patient 6	1	Loss	3,699,200	3,808,963	109,763	1p36.32	1p36.32
	1	Loss	7,493,277	10,233,944	2,740,667	1p36.23	1p36.22
	3	Loss	90,387,361	163,966,062	73,578,701	3p11.1	3q26.1
	3	Loss	163,997,258	164,101,806	104,548	3q26.1	3q26.1
	3	Loss	164,138,372	199,430,952	35,292,581	3q26.1	3q29
	8	Loss	39,356,625	39,499,782	143,157	8p11.23	8p11.23
	10	Gain	46,404,949	46,568,524	163,576	10q11.22	10q11.22
	17	Loss	77,564,147	78,562,684	998,537	17q25.3	17q25.3
	6	Loss	32,595,429	32,633,716	38,287	6p21.32	6p21.32
	8	Gain	39,356,625	39,499,782	143,157	8p11.23	8p11.23
Patient 9	4	Gain	69,085,413	69,165,843	80,43	4q13.2	4q13.2
Patient 10	13	Loss	74,901,500	74,998,123	96,623	13q22.2	13q22.2
	17	Loss	21,765,933	21,843,171	77,239	17p11.2	17p11.2
Patient 11	1	Gain	554,298	639,611	85,313	1p36.33	1p36.33
	4	Loss	4,912,553	4,913,217	664	4p16.2	4p16.2
	17	Loss	1,906,400	1,908,874	2,475	17p13.3	17p13.3
	19	Loss	1,329,394	1,346,556	17,163	19p13.3	19p13.3
	22	Loss	49,258,804	49,413,356	154,553	22q13.33	22q13.33
Patient 12	1	Gain	195,011,374	195,065,896	54,523	1q31.3	1q31.3
	7	Loss	26,424,077	26,541,278	117,201	7p15.2	7p15.2
	16	Loss	47,020,912	47,084,653	63,741	16q12.1	16q12.1
	17	Loss	38,158,239	38,202,311	44,072	17q21.31	17q21.31

DNA pool: DNA from homogeneous groups of patients. Chromosome: indicates the chromosome number according to the human karyotype. Type of aberration: loss—deletion of chromosomal material; gain—amplification of chromosomal material. Position in bp (start and end): indicates the first and the last base pair of the CNVs in the genome according to database of genomic variants. Size (bp): the size of CNV in base pairs. Band (start and end): the cyt locus of the CNVs in the chromosome according to human ideograms

YAP1, *BIRC3*, *BIRC2*, *TMEM123*, *MMP7*, *MMP20*, *MMP27*, *MMP8*, *MMP10*, *MMP1*, *MMP3*, *MMP12*, *MMP13*, *DCUN1D5*, *DYNC2H1*, *PDGFD*, *DDI1*). The presences of deletions in this region were found in 20 of 32 patients with cervical tumors [8]. It is assumed that this deletion occurs in the later stages of tumorigenesis and the affected genes are associated with cancer of the ovary, colon, skin, and breast [10, 20, 21].

The second pathogenical variation is a deletion in the short arm of the first chromosome (1p36.23-p36.23), 2,740,667 base pairs in size covering 28 gene (*CAMTA1*, *VAMP3*, *PER3*, *UTS2*, *TNFRSF9*, *PARK7*, *ERRF1*, *SLC45A1*, *RERE*, *ENO1*, *CA6*, *SLC2A7*, *SLC2A5*, *GPR157*, *MIRN34A*, *H6PD*, *SPSB1*, *SLC25A33*, *TMEM201*, *PIK3CD*, *C1orf200*, *CLSTN1*, *CTNNBIP1*, *LZIC*,

NMNAT1, *RBP7*, *UBE4B*, *KIF1B*). This deletion is common in glioblastoma tumors [5], breast cancer [3], lung cancer [20], colorectal cancer [4]. The deleted region contained *UBE4B* gene which is related with the rapid degradation of the product of tumor suppressor gene *p53* [23, 25]. Deletion in our case shows the direct link between tumorigenesis and deletion of this gene.

Pathogenical abnormalities were found in the same patient, as deletions in chromosome 3 (3p11.1q26.1, 3q26.1q26.1, 3q26.1q29). An interesting fact is that all three of them have a common aberrations band 3q26.1, area containing oncogenes (*SKIL*, *TERC*, *ECT2*). The losses of these vast regions of chromosome 3 indicate a late stage of tumorigenesis whereby genetic instability of the cellular DNA is extremely high [12, 13, 15].

The next three pathogenical abnormalities were detected in the microarray analysis of DNA of one patient. One is a deletion in the short arm of chromosome 4 (4r16.2r16.2) with size of 664 bp and covering 1 gene (*MSX1*). Literature data showed that deletions in this region are characteristic of patients with cervical intraepithelial neoplasia [18]. *MSX1* was specified as a possible candidate in the study to determine the early prognostic markers for breast cancer.

Final pathogenical aberration is a deletion in the long arm of chromosome 22 (22q13.33q13.33), 154,553 base pairs in size covering 14 genes (*SBF1*, *ADM2*, *MIOX*, *LMF2*, *NCAPH2*, *SCO2*, *TYMP*, *ODF3B*, *KLHDC7B*, *C22orf41*, *CPT1B*, *CHKB*, *MAPK8IP2*, *ARSA*). According to literature data *SCO2* plays a role in the regulation of the expression of *p53*, which plays a fundamental role in CC [19, 24].

The most common normal variations in our study were individually 8p11.23p11.23 and 4q13.2q13.2. The other two normal variations (6p21.32 deletion and duplication 10q11.22) established in our study were met only in one of the studied patients.

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