Chapter 13

Caspases as Putative Biomarkers of Cervical Cancer Development

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Additional information is available at the end of the chapter

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Abstract

Resistance to apoptosis is commonly accepted as the principal hallmark of a cancer cell, while caspases are recognized as the key molecular players of the apoptosis regulatory network. Since the level of caspase activity is thought to be directly coupled with aggressive features of cancer cells (such as ability to withstand immune reactions, invasiveness, drug resistance, etc.), these proteases could serve as objective diagnostic markers especially for those types of cancer where early differential diagnosis is needed. Cervical cancer develops through morphologically well-described stages—from intraepithelial lesions of 1/2/3 grade including carcinoma *in situ* to microinvasive and invasive cancer with precancerous lesions known to be potentially reversible. The percentage of cervical neoplasms diagnosed at early stages is relatively high, providing a basis for the use of cervical cancer as an *in vivo* model to investigate the mechanisms of apoptosis modulation in malignant cells. The existing diagnostic criteria, despite their usefulness, have substantial limitations with respect to cervical cancer and preneoplastic lesions, so caspases may be helpful in improving them, but there is insufficient data regarding the involvement of these enzymes in cervical cancer development. In this chapter, we report on specific patterns of activity of caspases revealed in tissue biopsies and blood lymphocytes in association with different stages of cervical cancer development. The data indicate that caspases are pivotal components of the *in vivo* molecular "portrait" of cervical cancer and have the potential of being used as biomarkers.

Keywords: Biomarkers of carcinogenesis, apoptosis, caspases, cervical cancer, human papillomavirus

1. Introduction

Carcinogenesis of solid tumors is a complex multistage process that generally develops over a long period of time through a succession of precancerous lesions. Cancer *in situ* and micro-

invasive cancer before the foci of tumor invasion and areas of metastatic growth are established. Until a certain step, the process is reversible—due to various intracellular control mechanisms and the immune system surveillance. Programmed cell death (PCD) represents the key mechanism for maintaining cellular homeostasis of a multicellular organism and eliminating abnormal potentially dangerous (transformed/virus-infected) cells that may be induced by either activation of the intracellular sensors of molecular abnormalities or by exposure to the cytotoxic factors secreted by immunocompetent cells. According to the concept of Hanahan and Weinberg [1], resistance to PCD inducers is a fundamental characteristic of a malignantly transformed cell. Various forms of PCD are presently well recognized with apoptosis still regarded as the major pathway. Apoptotic cell death is characterized by clearly defined morphological and biochemical changes and generally requires the involvement of a special class of intracellular proteases—the caspases (E.3.4.22).

In the process of carcinogenesis, the ability to inhibit apoptosis is believed to be established by gradual accumulation of mutations and/or epigenetic modifications of tumor suppressor genes with further selection of the most resistant cell clones. The expression levels of molecular components of the apoptotic signaling pathway and their functional activity reflect the degree of malignization at the site of a developing neoplasia. At the same time, in their research practice when studying the molecular mechanisms of apoptosis inhibition and searching for the ways of its reactivation, scientists usually deal with tumor cell lines and xenograft models that represent the final result of a long process of establishing the ability to resist apoptotic death and thus do not reproduce the complex multistep nature of its development to the full extent. The major reason for this situation lies in the fact that, for many types of cancer, the initial steps of carcinogenesis (precancer lesions, true noninvasive cancer, and microinvasive carcinoma) are very difficult or practically impossible to diagnose.

Cervical cancer (CC) exemplifies an oncopathology for which all the steps of carcinogenesis, including the earliest ones—cervical intraepithelial neoplasia (CIN) grades 1, 2, and 3 as well as microinvasive cancer— are described in sufficient detail relying on morphological criteria as opposed to many tumor types having other locations (Figure 1). Thanks to the successful implementation of the mass screening prevention programs, the percentage of cervical neoplasms diagnosed at early stages is notably higher than that of advanced stages representing another distinctive feature of CC. Precancerous (intraepithelial) lesions generally persist over a long period of time and, according to epidemiological evidence, undergo spontaneous regression with the high rate of frequency [2], but once a malignant phenotype is established, rapid invasion and fast dissemination are observed. Thus, the availability of a "set" of thoroughly defined and easily detectable stages of CC development gives enough reasons for considering CC as a unique *in vivo* model for studying the driving forces and the mechanisms of carcinogenesis, including pathways of PCD dysregulation. So far as the problem of PCD is concerned, the viral etiology of CC should be mentioned: human papillomavirus (HPV) is the cause of more than 99% of CC instances, and repression of both the differentiation program and apoptosis in an infected cell is thought to be the main way for the virus to implement its replicative strategy upon "productive infection," the question to be discussed in more detail in the main body of the chapter.

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Not only understanding of the fundamental significance but also promising opportunities for investigation of new diagnostic criteria and therapeutic tools can explain a constantly increasing interest to the use of CIN and CC as a natural model for the study of the apoptosis dysregulation pathways. The broad resistance of tumor cells to inducers of apoptosis remains one of the largest (if not the largest) obstacles to the effective implementation of the most of approaches currently being developed for radio-/chemo-/immunotherapy of human malignancies. CC is one of the most aggressive types of cancer as it is characterized by rapid acquisition of chemoradioresistance, fast progression, quick dissemination, and high recurrence rate even if diagnosed at the earliest stages. Despite the fact that CC is frequently diagnosed at early (including preclinical) stages, it still has one of the highest mortality rates, indicating insufficient level of our knowledge of the apoptosis inhibitory mechanisms involved in CC development and acute need of searching for the novel diagnostic biomarkers.

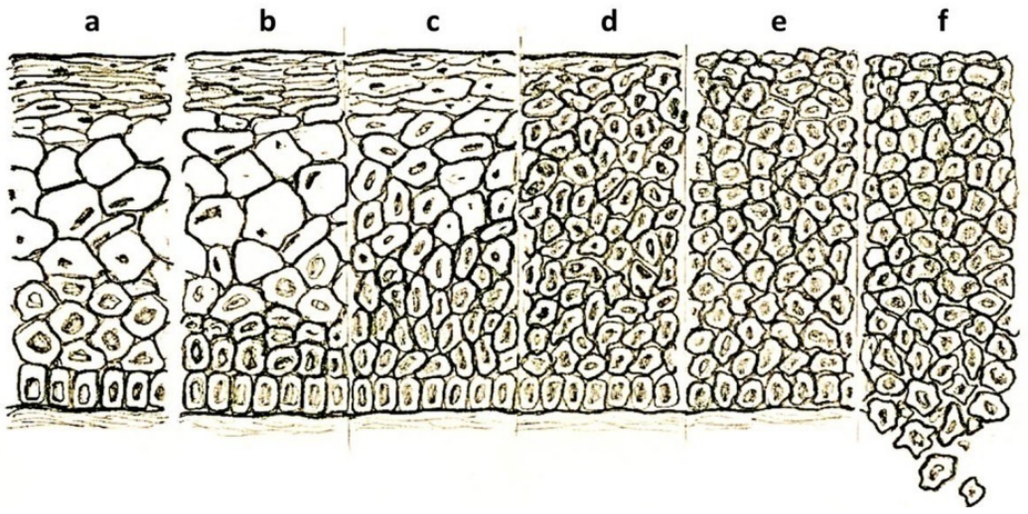

Figure 1. (a) Squamous cell epithelium of the cervix (the normal structure); (b–f) the continuum of cervical cancer development. Replication of HPV within the basal layer results in hyperplasia of parabasal cells encompassing no more than one-third of epithelium thickness and designated as CIN 1 (b). In CIN 2 (c), actively proliferating cells spread to the one-half of epithelial depth, while in CIN 3 (d–e), they constitute almost all the layers of cervical epithelium. Microinvasive cancer (f) is defined as a lesion in which neoplastic cells disrupt the basement membrane and invade the underlying stroma to a depth ≤3 mm.

Genome-wide studies have clearly demonstrated that cancerous cells, including cervical cancer cells, exploit a great variety of pathways in order to suppress or to bypass apoptosis. An important inference that can be drawn from these studies is that either the survival of a tumor cell or its death does not depend on the expression level of individual pro- and antiapoptotic factors but is rather determined by the ratio of activities of multiple apoptosis regulators (both activators and inhibitors), i.e., "molecular context." Caspases are those molecular players that eventually read the context, thereby implementing apoptosis-resistant or apoptosis-sensitive phenotype of a cell. On the one hand, caspases have been the central subject of research in the field of molecular oncology for many years but, on the other hand, analyzing the articles discussing the role and functional features of caspases in the natural

history of CIN and CC, one can come to a surprising conclusion that there is little information on this issue. The available data are, to some extent, conflicting and related, for the most part, to the artificial cell systems in which the level of caspases' activity/expression is often considered just as an indicator of experimental exposures. That is the reason why we have focused our attention on investigation of expression and functional activity of caspases at different steps of CC development. In the present chapter, we aimed to prove, relying on both our observations and published data, that caspases are the central targets for the action of antiapoptotic mechanisms driving the process of CC development. We also tried to show that the caspases activity pattern and the level of apoptosis in the fraction of blood lymphocytes may represent a valuable characteristic for estimating a patient's immune status in its response to different treatment approaches. Taking into account the specificity of changes revealed for different caspases and different steps of CC progression discussed in the chapter, we propose to consider caspases as promising biomarkers for cervical malignancies.

2. Body

2.1. Molecular mechanisms of cervical cancer development—interference of HPV oncogenes with apoptotic program of an infected cell

As was mentioned above, cervical cancer originates from persistent papillomavirus infection. In most cases, CC development is associated with the so-called high-risk HPV types, with HPV16 and HPV18 being the most common variants. The HPV genome has a small size and codes for several proteins, three of which—E6, E7, and E5—being regarded as proteins with oncogenic properties in view of their capacity to stimulate cell proliferation, convert cells into immortalized state, increase the frequency of mutations, and suppress apoptosis.

HPV displays a clearly defined tropism in relation to proliferating cells of the basal layer of squamous cervical epithelium since it is in these cells that the DNA replication "machinery" required for the virus to replicate itself is maintained in the active state. As basal keratinocytes move from the growth layer to the upper ones, they normally exit cell cycle and undergo terminal differentiation; however, this might impose limitations on the replicative potential of the virus. To evade this, the HPV oncoproteins provide conditions that enable infected cells to retain the ability to perform DNA synthesis even in the highly differentiated state ("productive HPV cycle"). The aberrant initiation of S-phase can occur owing to the E7-mediated degradation of pRb-protein releasing E2F transcription factor that triggers the expression of cyclins and other S-phase regulators. At the same time, in order to avoid p53-dependent apoptosis promoted by the uncontrolled cell cycle activation, E6 protein targets p53 for proteasomal degradation. Thus, the inhibition of apoptosis is an integral component of the HPV life cycle, which nevertheless does not lead to malignant transformation since proliferative and antiapoptotic activity of E6 and E7 is overcome by mechanisms of negative control of cell division [3]. At the morphological level, the progression of productive infection manifests itself in the form of hyperplasia of the deepest layers of epithelium, referred to as CIN 1 (see Figure 1). However, sometimes the HPV life cycle may take the path of "nonproductive infection,"

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followed by the overproduction of only two of the viral proteins, E6 and E7, but not resulting in the release of mature viral particles. An accidental transition of HPV from episomal state to the integrated one (happening presumably at CIN2/3 stage) is commonly accepted to be the main cause of dysregulation of the viral genome expression; however, the involvement of other mechanisms (e.g., epigenetic) is assumed as well. Anyway, the nonproductive infection can be considered as a by-product of HPV activity that significantly increases the probability of malignant transformation of cervical keratinocytes.

The mechanism of E6/E7-induced transformation is not confined exclusively to the degradation of the key cellular "guardians" pRb and p53. Recent studies have elucidated the existence of an intricate HPV interactome, i.e., a network of intermolecular interactions of E6 and E7 with the host cell proteins [4]. By these interactions, the E6 and E7 proteins can dramatically change the profile of gene activity, epigenomic landscape, and host cell proteome and modulate the majority of intracellular signaling pathways (including MAPK-, Wnt-, Akt-, Notch-, mTORC-, and STAT-dependent cascades), leading to virtually total reprogramming of an epithelial cell [2]. Degrading the p53 и pRb tumor suppressors, E6 and E7 remove cell cycle "checkpoints," favoring the gradual accumulation of somatic mutations. Moreover, E7 can impair the formation of the mitotic spindle causing large-scale chromosome aberrations and inducing genomic instability. At the phenotypic level, all of these molecular alterations facilitate acquisition of the properties referred to as "The 10 Hallmarks of Cancer" [1, 3], with the resistance to the programmed cell death, or apoptosis, being the basic one.

Molecular mechanisms of apoptosis have been thoroughly investigated in recent decades, with new endogenic factors exhibiting pro- and antiapoptotic activity being constantly discovered. A wide diversity of factors and receptors inducing apoptosis reactions in a cell were identified; however, almost all of the signaling paths were shown to converge, one way or another, to the common effector molecules—caspases. Caspases are a family of intracellular cysteine-dependent endoproteases produced in the form of nonactive proenzymes and, in case of receiving an appropriate signal, activated on the cascade principle. Eleven human caspases are presently known. The activation of caspases can proceed by the two major mechanisms: (1) oligomerization on specific "molecular platforms" and (2) partial proteolytic cleavage. The first mode of activation is typical for the so-called initiator caspases, which include caspases 2, 8, 9, and 10. The initiator caspases can accept extra- or intracellular signals and trigger "extrinsic" or "intrinsic" apoptotic pathway, respectively. The extrinsic pathway generally starts with the activation of a cell death receptor: binding of the receptor extracellular domain to its ligand brings its cytoplasmic part to the conformational change that then triggers the assembly of a membrane-associated macromolecular signaling complex (DISC). DISC serves as a "platform" for the recruitment and activation of the caspase 8 or 10 multimers. Several cell death receptors belonging to the Tumor Necrosis Factor (TNF) superfamily, namely, Fas/CD95/APO-1 (a prototypic member), TNF-R1, TRAIL-R1, TRAIL-R2, DR3, DR4, DR5, and DR6, have been discovered to date. In case of the intrinsic pathway of apoptosis, the apoptosome is such a molecular "platform" that supports the process of caspase 9 activation. The apoptosome complex assembly can be initiated in response to metabolic stress, disturbance of mitochondrial membrane integrity, or DNA damage. When activated, initiator caspases perform

processing of the downstream executioner (effector) caspases 3, 6, and 7, which, in turn, destroy numerous structural, regulatory, and catalytic intracellular proteins. Enzymatic activity of caspases is tightly regulated by various endogenic inhibitors and activators, including IAP-1/-2, Bcl-2 family members, Smac/DIABLO, c-FLIP, Survivin/BIRC5, XIAP, NAIP, livin, and others. The level of activity of caspases (and thereby susceptibility to apoptotic signals) is thought to be determined by the ratio of expression levels of their endogenous modulators. Many human diseases, including neoplasms, are known to be accompanied by the repression of caspases functions; however, this repression usually not associated with abnormalities in their gene structure that distinguishes caspases from the so-called tumor suppressors. Inactivation of caspases occurs in cancer cells as a consequence of the overexpression of their inhibitors or as a result of suppression of the upstream components of the apoptotic signaling pathways, as for example death receptors [5].

Due to active mutational processes and viral etiology, cervical cancer is characterized by particularly early and rapid development of resistance to apoptosis. Numerous articles reporting on the influence of different variants of post-transcriptional E6/E7 silencing on the cell sensitivity to the apoptosis inducers provide strong evidence that hyperexpression of HPV oncogenes is the primary cause and a prerequisite for the development and maintenance of apoptotic-resistant phenotype. For example, the transfection of E6-siRNAs (small interfering RNAs) into CC cells conferred susceptibility to cisplatin-induced apoptosis [6]. Similarly, the expression of an E7-targeted RNA-aptamer disrupting the interaction between E7 and pRb resulted in the induction of apoptosis [7]. Treatment with the synthetic peptide anti-E7 antagonist was shown to suppress tumor growth in an animal xenograft model due to activation of apoptotic cell death [8]. Analysis of the published data revealed three basic groups of mechanisms that modulate apoptosis signaling pathways by engagement of HPV oncoproteins: (1) inactivation of proapoptotic proteins resulting from direct binding to E6/E7 with subsequent ubiquitination and proteasome-mediated degradation; (2) interactions of E6/E7 proteins with the cellular transcription factors and chromatin-remodeling enzymes leading to the change of either the mRNA expression pattern of pro- and antiapoptotic factors, or the profile of microRNAs (miRNAs) targeted these factors; and (3) HPV oncogene-mediated induction of genomic instability that causes either the accumulation of inactivating mutations in the proapoptotic oncosuppressor genes, or, alternatively, the amplification of antiapoptotic genes.

1. "High-risk" HPV oncoproteins are capable of high-affinity binding to various protein components of the extrinsic or intrinsic apoptosis pathways and stimulating their degradation due to the ubiquitin-ligase activity, thereby blocking signal transduction from an apoptogenic stimulus. In HPV-positive cells, the membrane expression of CD95/Fas, the key cell death receptor, is significantly reduced, and the DISC assembling is impaired because of accelerated destruction of the FADD adaptor protein and caspase 8, with endogenous inducers of mitochondrial apoptosis pathway (such as Bid, Bak, and Bax antagonists of Bcl-2 protein) being degraded as well (for a review, see [9, 10]). That is why the abrogation of proteasome functions (by MG132 or Bortezomib treatment, for example) potentiates the activity of caspases and sensitizes CC cells to TRAIL-/Fas-dependent apoptosis or radiation-induced cell death [11]. E5 protein, similarly to E6 and E7, can impair the mechanisms of CD95L- and TRAIL-mediated apoptosis [10, 12].

2. Epigenetic modification of apoptotic genes is one of the mechanisms of global regulation of the cell death program in cervical cancer. HPV has been shown to dramatically alter the host DNA methylation landscape, especially within the promoter regions of tumor suppressor genes and genes coding for apoptosis activator proteins, thus facilitating conversion of these genes into the heterochromatin state. For example, the promoter hypermethylation of PRDM14 gene encoding a transcription factor which is required for the expression of NOXA and PUMA proapoptotic regulators of Bcl2-family was observed in HPV16-bearing cell lines and primary tumors [13]. Histone acetylation/deacetylation is another way of modulating gene activity employed by CC cells. The treatment of CC cell lines with various histone deacetylase inhibitors results in apoptosis induction followed by activation of caspases 3, 8, and 9, PARP cleavage, and loss of mitochondrial membrane potential, thus confirming the importance of this epigenetic mechanism for the establishment of apoptotic resistance [14–16]. In a similar manner, silencing of the MLL5β histone methyltransferase has an apoptosis-inducing effect on CC cells [17]. Transcriptomic studies also suggest that among the different functional groups of genes whose expression is affected by the presence of HPV oncogenes, the apoptosis-regulatory genes (as for example, BCL2, BCLXL, and c-IAP1 [18]) constitute a substantial portion. The expression profile of pro- and antiapoptotic miRNAs in CC cells also arouses much interest among researchers. More than 100 miRNA species were documented to change their expression in the presence of the viral E6/E7 proteins, and many of their mRNA-targets were found to code for various regulators of apoptosis and, in particular, caspases, as for example survivin and Bcl-family proteins [19–22]. The novel long noncoding RNAs (lncRNAs) contributing to the development of apoptosis-resistant phenotype of CC cells have been described as well [23].

3. In cervical cancer cells, the deletions of the chromosome loci containing genes required for the apoptotic program to be implemented are found to occur at a high frequency [24, 25]. Although having sporadic nature of occurrence, these genetic abnormalities most likely confer a selective advantage to cancer cells for further expansion that probably explains why the incidence of such abnormalities increases with tumor progression [24]. In contrast, for genes encoding inhibitors of apoptosis, amplification of the corresponding genome segments is frequently observed with CC progression [26].

As evidenced by the above-stated examples, the mechanisms cervical cancer cells employ to achieve apoptotic resistance engage all the levels of intracellular regulation—genomic, transcriptomic, epigenomic, and proteomic. Although all the diversity of the known molecular pathways is ultimately directed to the suppression of caspases as the crucial mediators of cell death reactions, the experimental data showing that their proteolytic activity does undergo specific changes in the natural history of cervical neoplasms appeared to be virtually absent in literature, thus prompting us to conduct research whose results are described below.

2.2. Induction of apoptosis in immunocompetent cells as a putative factor of cervical cancer progression

Because of the viral etiology of cervical cancer, the mechanisms of its development need to be investigated in conjunction with changes occurring in the immune system. In each individual

case, it is not only the properties of tumor cells (the mutation spectrum, the gene expression profile) that determine the progression of CIN to invasive metastatic state but also the survival and growth of secondary tumor foci. The abilities of the immune system to recognize and eliminate virus-infected and malignantly transformed cells are believed to be of great importance too. According to the general conception, it is due to the reactions of the immune system that both the HPV infection and dysplastic alterations of squamous epithelium (CIN1/2) are usually transient, and there is only a small percent of cases that develop to chronic or malignant form [2]. On the other hand, various mechanisms exploited by the virus and/or tumor cells for specific inhibition or avoidance of immune reactions are becoming elucidated in the last years. Furthermore, possible involvement of supplementary "environmental" factors exerting suppressive influence on the immune system of an organism is assumed. The induction of apoptosis in immunocompetent cells is supposed to be one of the mechanisms to inhibit antitumor/antiviral immunity. Although there is some evidence of increased expression of apoptosis-related markers in tumor-infiltrating or circulating lymphocytes of cancer patients, in case of cervical cancer, such information is scarce [27, 28]. CC cell lines as well as primary CC cells are known to express CD95L (FasL) [29] and, when cocultured, to induce apoptotic death in cytotoxic T-lymphocytes, the effect being abolished by anti-CD95 antibodies [30]. It was also found that treatment of peripheral blood lymphocytes taken from healthy donors with conditioned media from CC cell lines could induce apoptosis in subpopulation of CD4+ T-helpers [31]. Reasoning from these facts, we hypothesized that during its *in vivo* development cervical cancer may withstand immune reactions via promoting apoptosis in effector immune cells, and specific change of activity/expression of caspase may therefore be detected in circulating lymphocytes of CC patients.

2.3. Materials and methods

2.3.1. Patients and samples

Tissue samples and peripheral blood were obtained from 156 patients who underwent surgery in Oncological Dispensary of the Republic of Karelia: 75 women diagnosed with cervical intraepithelial neoplasia grade 3 (CIN 3, with average age at diagnosis 32.9 ± 7.4 years) and 81 women with squamous carcinoma, including 45 with stage IA (average age 31.3 ± 6.0), 21 with stage II (average age 43.6 ± 13.2), and 15 with stages III–IV (average age 46.9 ± 11.1), were examined. Stages of cancer were defined in accordance with the TNM-classification and the International Federation of Gynecology and Obstetrics system (FIGO, 1994). CIN 3 and CC diagnosis was based on comprehensive physical examination, extended colposcopy findings, cytology, and histopathology tests, in full compliance with the approved standards for the diagnosis and treatment of patients with gynecological malignancies. All women enrolled in this study were informed and gave voluntary written consent. The research was approved by the Committee on Medical Ethics of Petrozavodsk State University and the Ministry of Healthcare and Social Development of the Republic of Karelia.

Extended colposcopy was performed in each case before surgery in order to define localization and precise margins of a lesion for subsequent accurate excision of tissue fragments.

Colposcopic findings were evaluated according to the International Federation for Cervical Pathology and Colposcopy (IFCPC, 2002) terminology. Tissue samples were obtained during cervical conization or total hysterectomy. In each case, two pieces of tissue were resected from the pathologic locus, which was defined both visually and colposcopically, and one piece of morphologically normal epithelium (control) was excised from the contralateral side of the cervix outside the pathologic zone. Tissue samples were immediately submerged into RNA-stabilizing solution RNALater (Qiagen) or RPMI-1640 medium (Gibco), then frozen and stored at −80°C. For all patients the original diagnosis was verified by histomorphological examination.

Venous blood sampling was done right before the surgery or any other treatment. The fraction of peripheral blood mononuclear cells (PBMC) was isolated by standard procedure in Ficoll density gradient (Paneco, Russia). Forty-five samples of peripheral blood were also taken from healthy nonpregnant HPV-negative women comparable in age and anamnesis, with no pathology of the cervix (control blood group 1, age characteristics: 23.4 ± 0.9 (n = 15); 33.3 ± 1.7 (n = 15); 46.7 ± 11.1 (n = 15)). For patients with CIN 3 and CC stage IA, blood samples were again collected in 1 and 3 months after conization and course of immunomodulatory therapy. Control group 2 consisted of patients with CIN 3 (n = 15) and CC stage IA (n = 15) (average age 34.1 ± 7.2) who underwent only surgical treatment. Patients of the examined groups did not differ in anamnesis, virological, and histological findings.

Screening for the presence of HPV DNA and identification of HPV genotype were performed by polymerase chain reaction (PCR) using AmpliSens HPV HCR Screen kit (The Central Research Institute of Epidemiology of The Federal Service on Customers' Rights Protection and Human Well-being Surveillance, Russia) and TaqMan probes. E6/E7 oncogene mRNA was detected by the reverse transcription coupled PCR (RT-PCR), and reagents kits were purchased from DNA-Technology and Sileks companies (Russia). The PCR products were visualized by 2% agarose gel electrophoresis. The distribution of HPV genotypes is displayed in Table 1.

	HPV16	**HPV16, 18, 31, 33**	**HPV18**	**HPV31**	**HPV33**
CIN 3	52.1	34.8	13.1	-	-
CC stage IA	79.4	-	11.8	5.9	2.9
CIN 3 + CC stage IA	61.2	23.3	12.6	1.9	1.0
CIN 3 + CC stage IA (3 months after treatment)	1.1	2.2	1.1	-	-

Table 1. The distribution (%) of HPV genotypes in groups of patients diagnosed with CIN 3 or CC of stage IA.

2.3.2. Real-time PCR

Total RNA from tumor cells and PBMC was extracted with TRizol reagent (Invitrogen, USA) following the manufacturer's guidelines. The concentration and purity of the RNA template was determined by spectrophotometry (BioWave II+, Biochrom, UK). RNA nativity was

determined by capillary gel electrophoresis using Experion Automated Station and RNA StdSens analysis kit (Bio-Rad, USA). The extracted RNA template was treated with DNase I (Fermentas, ThermoScientific, USA). Complementary DNA (cDNA) was synthesized from 1 μg of total RNA using random hexaprimers and ProtoScript MMLV reverse transcriptase following the protocol proposed by the manufacturer (New England BioLabs, UK). RNA and cDNA samples were stored at −80°C. Gene expression was estimated by SYBR Green real-time PCR. Amplification was performed in StepOnePlus thermal cycler (Applied Biosystems, USA) with StepOne™ Software v2.2.2 using 20 ng of cDNA per 1 reaction volume (25 μl) and qPCRmix-HS-SYBR+HighROX 5×-reaction mix (Evrogen, Russia), containing gene-specific primers at final concentration 0.5 μM. Primers for the nucleotide sequences of the investigated genes were selected from published sources (Table 2). Oligonucleotides were synthesized by the Evrogen company (Russia). The PCR protocol was 20 s at 95°C, 20 s at 60°C, and 30 s at 72°C (45 cycles). All the reactions were done in duplicates. Effectiveness of amplification (%) was monitored by standard curve approach. To determine the specificity of primer annealing, the PCR fragments were melted: 1 min at 95°C, 1 min at 60°C, and 10 s at 60°C (80 cycles, the temperature raised by 0.5°C in each cycle). To exclude the possibility of the template cDNA being contaminated by the genomic DNA, PCR was performed for each template under the same conditions with the RNA matrix (negative control). The resultant reaction products were also separated in 8% polyacrylamide gel using the Tris–borate buffer, then stained with 1% ethidium bromide solution and visualized in transmitted UV light (with GelDoc-It Imaging System, UVP, USA). The correspondence of amplicon sizes to theoretically expected ones was confirmed using the low-molecular pUC19/Msp I fragment length marker (Syntol, Russia). Gene mRNA expression was measured using the $2^{-\Delta\Delta Ct}$ method [32]. cDNA samples from normal epithelial tissue or from PBMC of healthy donor were used as calibrator.

Gene	Sequence	PCR product length	Source
GAPDH F	5′-GAAGGTGAAGGTCGGAGTC-3′	225	[33]
GAPDH R	5′-GAAGATGGTGATGGGATTTC-3′		
Caspase 6 F	5′-ACTGGCTTGTTCAAAGG-3′	181	[34]
Caspase 6 R	5′-CAGCGTGTAAACGGAG-3′		
Caspase 3 F	5′-ATGGAAGCGAATCAATGGAC-3′	240	[35]
Caspase 3 R	5′-ATCACGCATCAATTCCACAA-3′		
Caspase 9 F	5′-AACAGGCAAGCAGCAAAGTT-3′	246	[35]
Caspase 9 R	5′-CACGGCAGAAGTTCACATTG-3′		

Table 2. Primers for the nucleotide sequences of the genes under study.

2.3.3. The enzyme activity of caspases

The enzyme activity of caspases was determined by standard technique using specific substrates labeled with fluorescent marker (7-amino-4-trifluoromethylcumarin—AFC) (Bio-

Rad, USA), detected by variations in fluorescence or optical density [36]. Fifty microliters of lytic buffer prepared by mixing 920 μl of bidistilled H_2O, 40 μl of 25-fold reaction buffer, and 10 μl of each of the four inhibitors: phenylmethylsulfonyl fluoride (PMSF) (35 mg/ml), pepstatin A (1 mg/ml), aprotinin (1 mg/ml), and leupeptin (1 mg/ml), were added to the tumor sample (5 mg) or PBMC (10^6 cells). The 25-fold reaction buffer included the following components: 250 mM HEPES, pH 7.4, 50 mM EDTA, 2.5% 3-((3-chloramidopropyl)dimethylammonio)-1-propanesulfonate (CHAPS), and 125 mM dithiothreitol. After that, the cells were frozen three times in liquid nitrogen, the cell lysate then centrifuged in a microcentrifuge at 17,000*g* (4°C) for 30 min, and the supernatant (template) collected. The activity of caspases 3, 6, and 9 was determined in the reaction buffer by mixing the template with the corresponding specific substrate. The substrate for caspase 3 was DEVD (Asp–Glu–Val–Asp), for caspase 6—VEID (Val–Glu–Ile–Asp), for caspase 8—LETD (Leu–Glu–Thr–Asp), and for caspase 9—LEHD (Leu–Glu–His–Asp). The amount of cleaved AFC was measured by spectrophotometry in FluoroMax ("Horiba-Scientific," Japan) at 395 nm 30, 60, 90, 120, 150, and 180 min after the onset of the reaction. Then, the curve of caspase activity depending on the template and substrate incubation time was plotted. Relative proteolytic activity was calculated as the slope $\Delta S/\Delta t$, where

$$\Delta S = \left[S\left(t_i\right) - B\left(t_i\right)\right] - \left[S\left(t_0\right) - B\left(t_0\right)\right],\ \Delta t = \left(t_i - t_0\right),$$

where S is the sample signal at time t, and B is the blank signal at time t, t_i is the time of measurement, and t_0 is the time of initial measurement.

2.3.4. Flow cytometry

Total leukocyte fraction was prepared via using ammonium chloride osmotic shock (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA), and 100 μl of whole blood was taken for each probe. Cells were centrifuged and washed with Versene solution (0.02% Na_2EDTA in phosphate-buffered saline). To measure the surface expression of CD antigens, the cells were probed with fluorophore-conjugated monoclonal antibodies (mAbs): CD3-APC, CD4-FITC, CD8-FITC, and CD95-RPE (Dako, Denmark). One hundred microliters of cell suspension was incubated with mAbs (1:20) for 30 min at room temperature. To prevent nonspecific Fc receptor-mediated mAb binding, the FcR Blocking Reagent was used (Miltenyi Biotec, Germany) in accordance with the manufacturer's instructions. Analysis was performed with MACSQuant Analyzer flow cytometer (Miltenyi Biotec.). Figure 2 describes the scheme for discriminating cell subpopulations of interest (the gating strategy); lymphocytes were gated by forward and side scatter. The numbers of lymphocytes having the following phenotypes were evaluated by the fluorescence parameters: $CD3^+$ (T-lymphocytes), $CD3^+CD4^+$ (T-helpers), $CD3^+CD8^+$ (T-killers), $CD3^+CD95^+$, $CD3^+CD95^{high}$, $CD3^+CD4^+CD95^+$, $CD3^+CD8^+CD95^+$, $CD3^+CD4^+CD95^{high}$, and $CD3^+CD8^+CD95^{high}$. Not less than 100,000 cells were analyzed in each probe. Dead cells were excluded from analysis by propidium iodide staining.

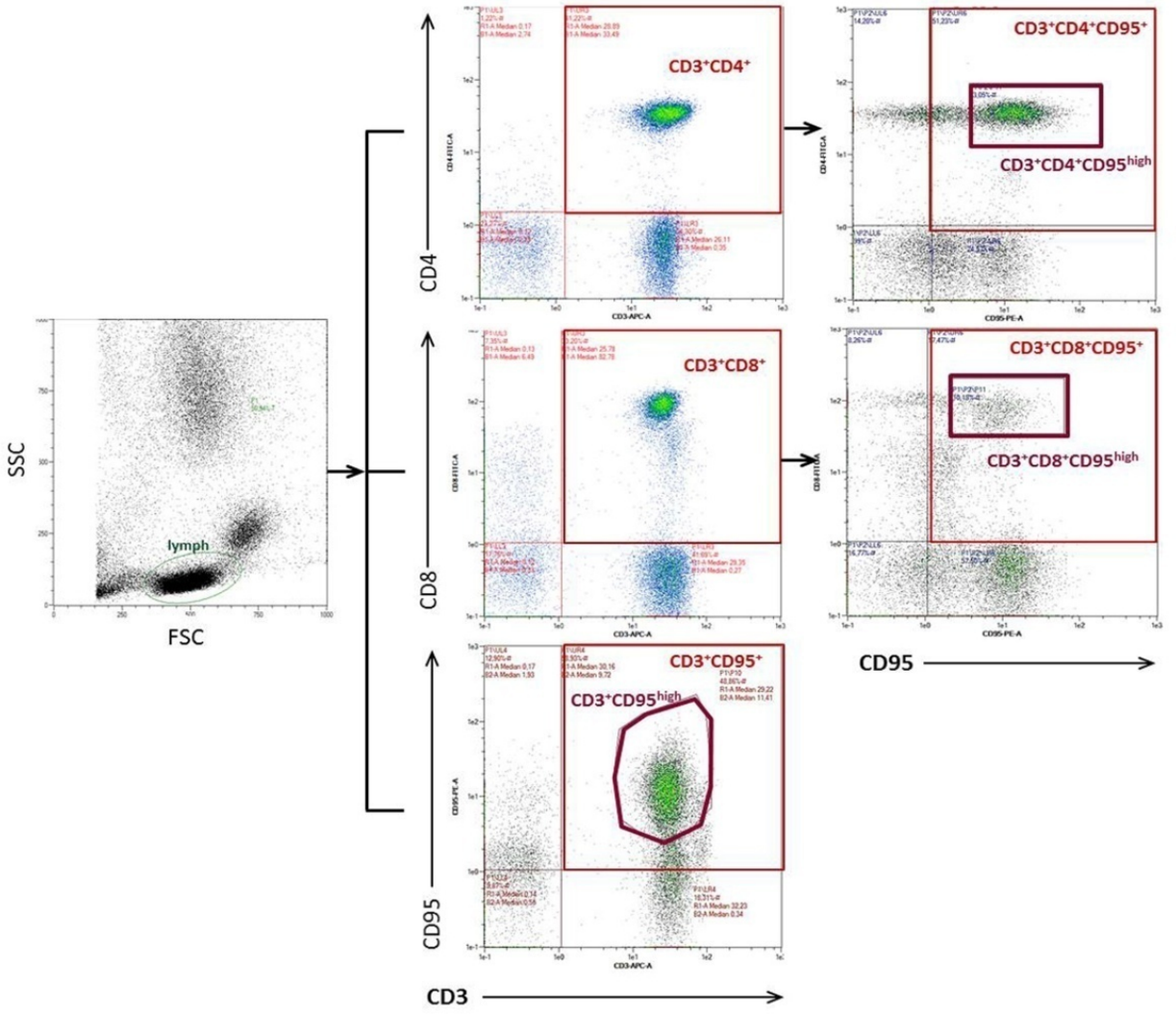

Figure 2. The scheme of the flow cytometric analysis of peripheral blood lymphocytes. Statistical reliability of the obtained results was estimated using the Student *t*-test and the nonparametric Wilcoxon–Mann–Whitney test. One-way ANOVA was also performed to compare between control/cancer groups of samples.

2.4. Results and discussion

2.4.1. Expression and activity of caspases in cervical intraepithelial neoplasia grade 3 and cervical cancer tissue samples

In the first phase of our research, we assessed the level of the relative mRNA expression of the initiator caspase 9 and executioner caspases 3 and 6 at different steps of CC development, starting from CIN 3 to advanced cancer (stages II–IV) comparing to the normal epithelium. The results are summarized in Table 3. Substantial fraction of CIN 3 samples (more than 50%) displayed the control level of caspases 3, 6, and 9 expressions, while the downregulation of caspase 3 and caspase 9 mRNAs was observed in the one-third of CIN 3 samples; caspase 6 was downregulated in 20% of samples. In the minor portion of CIN 3 cases (less than 20%),

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the relative amount of caspase 3, caspase 6, and caspase 9 transcripts was increased compared to the control. Microinvasive cancer (stage IA1) showed elevated mRNA levels for all three caspases in 50% of cases, with other cases displaying no difference or decrease (of various extents) relative to the control level. Analysis of stage II–IV CC samples revealed that mRNA levels of caspases 3, 6, and 9 generally did not differ from those of normal epithelium. For the rest of the advanced cancer samples, the upregulation of caspases 3 and 6 was detected, with caspase 9 showing inverse relation. In summary, cervical cancer progression was not found to be significantly correlated with mRNA expression of caspases 3, 6, and 9.

	CIN 3 (n = 25)	CC stage IA (n = 12)	CC stage II–IV (n = 10)
Caspase 3	Control, 56% (*n* = 14)	Control, 33% (*n* = 4)	Control, 60% (*n* = 6)
	↑ , 16% (*n* = 4)	↑ , 50% (*n* = 6)	↑ , 40% (*n* = 4)
	↓ , 28% (*n* = 7)	↓ , 17% (*n* = 2)	
Caspase 6	Control, 60% (*n* = 15)	Control, 25% (*n* = 3)	Control, 70% (*n* = 7)
	↑ , 20% (*n* = 5)	↑ , 50% (*n* = 6)	↑ , 30% (*n* = 3)
	↓ , 20% (*n* = 5)	↓ , 25% (*n* = 3)	
Caspase 9	Control, 52% (*n* = 13)	Control, 42% (*n* = 5)	Control, 40% (*n* = 4)
	↑ , 16% (*n* = 4)	↑ , 50% (*n* = 6)	↑ , 60% (*n* = 6)
	↓ , 32% (*n* = 8)	↓ , 8% (*n* = 1)	

Table 3. The change of mRNA levels of caspases at CIN 3 → CC progression relative to the normal epithelium. Arrows (↑ or ↓) correspond to up- or downregulation relative to the control level.

As modulation of caspases proteolytic activity is regarded as the highest level of regulation, we further explored its relative change in normal epithelium, CIN 3, and invasive CC specimens using specific tetrapeptide fluorescently labeled substrates. In 38% of CIN 3 samples tested, caspases 3 and 6 exhibited increased activity compared to the control level, with caspase 9 activity being upregulated only in a few (14%) CIN 3 cases (Figure 3). As for the rest of CIN 3 samples, the protease activity of caspases was found to diminish or correspond to the control level. In those CIN 3 samples that exhibited altered caspase 3 activity, either downregulation or upregulation was significant as compared to the normal epithelium (Figure 3A). In contrast to caspase 3, the activity of caspase 6 matched the range of control values in 50% of CIN 3 cases; the other 50% of tissue samples demonstrating significant (2.5- to 3-fold) increase in caspase 6 activity (Figure 3B). The activity of caspase 9 in CIN 3 group was generally comparable to that of the control group (Figure 3C). Stage IA was characterized by significant decrease of activity of all caspases analyzed (for caspases 3 and 9, the median activity values were significantly lower than those of the control samples); the same trend was observed for stages II–IV of CC. Note that in 23% of CC stage IA samples, the activity of caspase 6 was notably increased.

To summarize, a gradual downregulation of caspases 3, 6, and 9 occurs as invasive CC progresses, while at the stage of preinvasive cancer (CIN 3) the activity of caspases 3 and 6 may be significantly higher than that of normal epithelium. This observation may indicate, in respect of apoptosis-associated processes, a high degree of molecular heterogeneity of lesions morphologically defined as CIN 3. The activity of caspases 3 and 9 was shown to be signifi-

cantly correlated with the clinical stage ($r = –0.72$, $R^2 = 0.52$, $p < 0.01$ for caspase 3; $r = –0.67$, $R^2 = 0.45$, $p < 0.01$ for caspase 9; linear regression). The correlation between caspase 6 activity and CC stage was not proved to be statistically significant. Comparing the results on the expression level of caspases 3, 6, and 9 mRNA, on the one side, and the change of their protease activity, on the other side, it can be inferred that, for the invasive forms of CC, these two regulatory levels (i.e., transcriptional and translational) are poorly correlated: as the activity of caspases decreases, their mRNA levels stay within the range of control values or increase. At the same time, mRNA expression and protease activity values were comparable with respect to CIN 3 lesions.

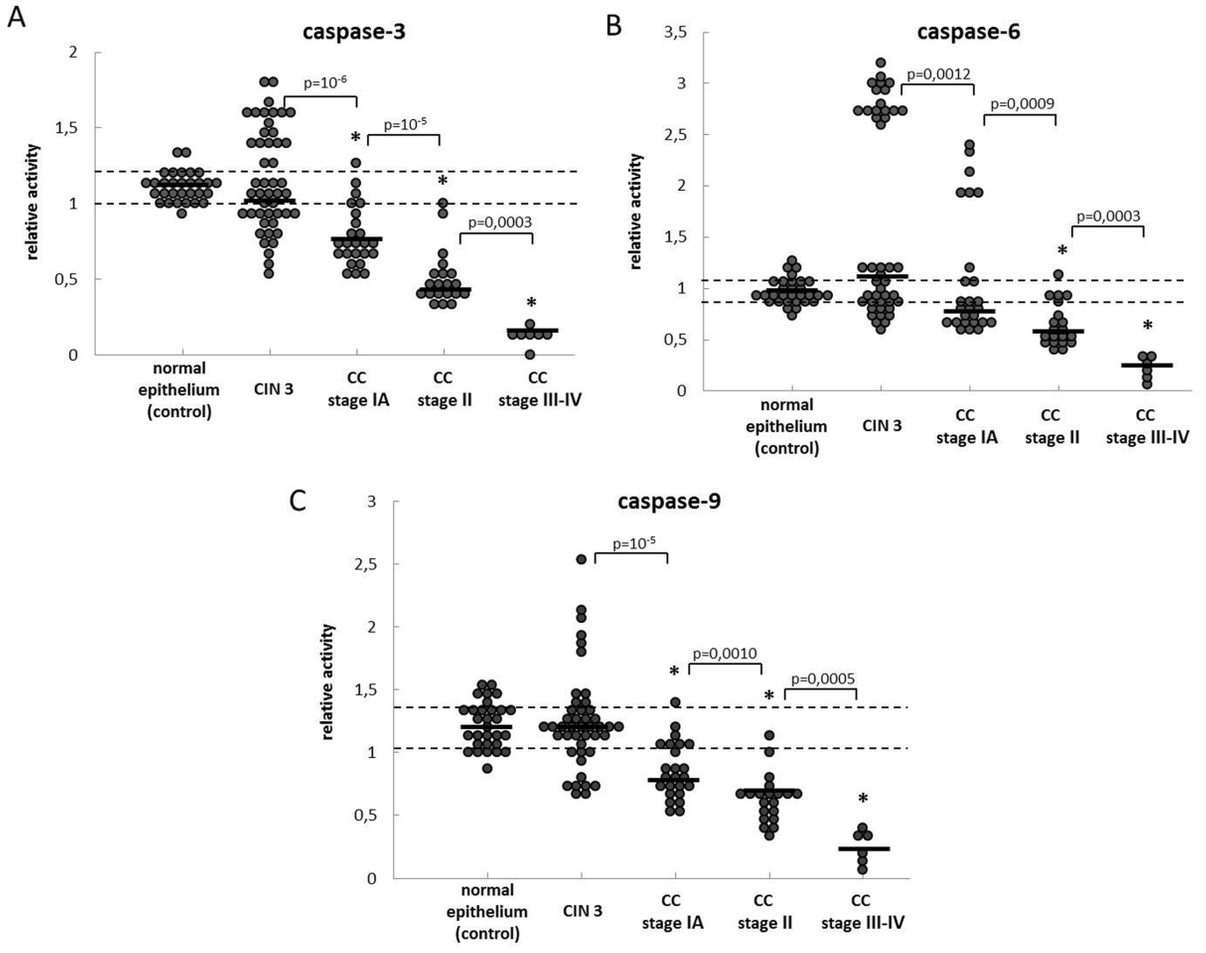

Figure 3. Scatter plots showing individual patients' changes of protease activity of caspases 3(A), 6(B), and 9(C) in pathological tissue at different stages of CC development. Horizontal bars represent the median activity level for each data set; dashed lines indicate ± standard deviation (SD) of the mean value in samples of normal epithelium (control). The results were multiplied by 10^4 for convenience. *Patient groups with median caspase activity being significantly different from that of control (the differences were considered significant if *p*-value was <0.05, *U*-test). The *p*-values shown on the plots were obtained by comparing successive stages of CC.

The above data allow us to speculate that the growth and development of CC is accompanied by multifaceted impairments of apoptotic processes implemented in the form of global suppression of caspases' functioning and multiple resistance of tumor cells to apoptotic stimuli. To our knowledge, this is the first systematic study investigating the changes of

expression/activity of functionally different caspases at different clinical stages of cervical cancer. Caspase 3 is a universal executioner caspase of both "extrinsic" and "intrinsic" apoptotic pathways. Caspase 6 represents one of the least studied members of the family, although new evidence indicates its crucial role in amplification of apoptotic signal; as distinct from other executioner caspases (3 and 7), caspase 6 is capable of being autoactivated. However, its elevated activity does not necessarily result in apoptosis [37]. It was also revealed that the range of caspase 6 targets does not overlap with that of other effector caspases [38]. Caspase 9 is a crucial intracellular sensor of apoptogenic stimuli such as hypoxia, genome destabilization, redox dysregulation, deficiency of prosurvival, and growth factors—all these stimuli inevitably affect the process of tumor development as a consequence of rapid accumulation of cell mass and overexpression of HPV oncogenes. Therefore, the suppression of caspase 9 is required to escape their influence. It is worthy of notice that the inhibition of caspase activity is an early event in CC progression since it was observed in the majority of stage IA1 samples (i.e., invasive microcarcinoma) and apparently reflects the establishment of an aggressive phenotype of CC cells.

The principal conclusion that can be made on the basis of our study and data available from literature is that caspases occupy the key position in the multistep model of natural CC development and have prospects of being used as biomarkers for this type of oncopathology. The importance of studying the profile of expression/protease activity of caspases with the use of cervical tissue samples is emphasized in the work of Arechaga-Ocampo et al. [39], where the authors come to a conclusion that the activity of caspases upon CC development is determined by the individual spectrum of genomic aberrations and the microenvironment. Therefore, the information on caspases expression patterns derived by the use of model cell systems describes isolated cases rather than reflects the *in vivo* situation. In view of this aspect, the elevated levels of activity of caspases 3 and 6 that we observed in CIN 3 samples seem to be of a particular interest. Indeed, there are some published reports arguing for frequent upregulation of apoptotic markers (caspases, internucleosomal DNA fragmentation) in clinical tissue specimens of CIN 3 [40–44], but nevertheless, the data pointing at the absence of such alterations should also be taken into consideration [45]. At this point, it is difficult to judge objectively whether the increase of caspase activity is linked to the higher risk of a subsequent fast progression of CIN to invasive cancer or, in contrast, the probability of long-term disease persistence without signs of invasive growth. To understand the biological significance of certain changes in activity of caspases and the driving forces underlying these changes upon CC development, it is necessary to continue research with inclusion of other regulatory molecular factors. Caspases as being multifunctional proteinases may participate in some processes that rule the HPV life cycle progression [46]. In particular, there is evidence showing that production of E-proteins is coupled with the increase of stability and activity level of caspases [47]. It was also discovered that HPV oncoproteins contain specific sites for caspase-dependent proteolysis [48]. The study of Moody et al. [46], carried on with the use of organotypic raft model of stratified epithelium, demonstrates the potential role for the proteolytic activity of caspases in the HPV life cycle. According to Moody et al., in raft cultures generated from normal keratinocytes, the active (processed) form of caspase 3 was present in small amounts and was concentrated predominantly in the basal cell layer. In similar cultures

composed of HPV31-infected keratinocytes, the active form of caspase 3 could be detected in all layers of epithelium; however, these two model cell systems revealed no difference in the amount of inactive caspase 3 (procaspase 3). Taken together, these data seem to be in poor agreement with the idea that the main influence of HPV oncoproteins is directed to the promotion of S-phase and inhibition of apoptosis. However, as argued by the authors [46], the high content of the activated form of caspase 3 observed in HPV-positive raft cultures was not coupled with any of the morphological features of apoptotic death. Moody and coauthors [46] proved that in HPV-positive cells, the activity of caspases, although being elevated, could not reach a "barrier" value sufficient to switch on the program of apoptosis. The HPV proteins presumably provide high threshold of sensitivity to the caspase-mediated reactions through potentiating the expression of antiapoptotic proteins—Bcl-2, survivin, IAPs, as well as other factors. The intricacy of mechanisms of shifting the balance between pro- and antiapoptotic regulators becomes even more evident in the light of new facts about apoptogenic effect of the E2F transcription factor: as it was mentioned earlier, hyperexpression of E7-oncogene liberates E2F, whose targets, along with proliferative genes, comprise genes encoding proapoptotic proteins Puma, Noxa, and Bim [4].

Indeed, in recent years, researchers pay much attention to the expression of endogenic inhibitors of caspase activity in cervical neoplasia and cervical cancer in view of the problem of multiple resistance of CC to programmed death. Such inhibitors are, for example, the members of IAP-family—XIAP and survivin. XIAP is known for its ability to bind caspases 3, 8, and 9, thereby preventing their activation, and, owing to its ubiquitin-ligase activity, to target proapoptotic factors for proteasomal degradation. The upregulation of XIAP has been documented in CIN and CC samples [49]; the involvement of XIAP in the maintenance of CC resistance to cisplatin and doxorubicin has been discussed as well [50]. Similarly, the disease progression occurred to be associated with upregulation of survivin that is able to inactivate caspases 3, 7, and 9 via direct binding to them [29, 51, 52]. The study of Lu et al. [53] revealed the negative correlation between survivin and caspase 3 expression levels in CC specimens by immunohistochemistry. In the work performed by Cao et al. [51], it is proposed to consider the survivin expression level as a marker enabling evaluation of the risk of CIN progression to invasive cancer. As is reported by Espinosa et al. [54], it is survivin that becomes overexpressed with CC stage progression, while other IAP members generally exhibit reduced expression in order to compensate survivin levels (the existence of such a compensatory relationship between the IAP-family members was established by experimental cell systems). Measuring survivin and caspase 3 expressions in both primary tumors and CC metastatic loci allows the prediction of a patient's response to chemotherapy (used in adjuvant or neoadjuvant settings) [53, 55, 56]. Closely, homologues to survivin is the recently discovered IAPs member —a livin protein [57] that was shown, similar to survivin, to be upregulated in cervical neoplasms and revealed to be negatively correlated with the level of caspase 3 expression [58]. There are some other inhibitors of the caspase-mediated pathway known to function by mechanisms distinct from IAPs, as for example c-FLIP. Interacting with FADD, c-FLIP prevents from caspase 8 recruitment and interferes with DISC formation upon activation of death receptor; an apoptosis inhibitory complex (AIC) is formed instead, thus prohibiting initiation of the caspase cascade [59]. The upregulation of c-FLIP in CIN and CC biopsies was also detected by several research groups [60, 61]. The role of c-FLIP in the development of CC

polyresistance to the wide range of proapoptotic agents is being investigated in parallel with the search of approaches to abrogate its function; for instance, it has been recently discovered that histone-deacetylase inhibitors can induce transcriptional repression of c-FLIP gene thus governing sensitization of CC cells to apoptosis [62]. In contrast to the protein inhibitors of apoptosis mentioned above, endogenic antagonists of IAPs (as for example, Smac/DIABLO) are poorly studied in CC, although their intracellular amounts may appear as important for determining the degree of CC cells' resistance/sensitivity to apoptosis as that of IAPs.

Thus, the reasons and the mechanisms that might account for the observed changes of the caspase activity pattern in the course of CC development appear to be quite diverse. The need to decipher these regulatory interrelationships is determined by the search for solutions to restore the functionality of apoptotic cascade, to increase the effectiveness of chemotherapeutic agents, and to overcome multidrug resistance of CC cells. For example, AT-406, a low molecular weight compound that imitates the binding of Smac/DIABLO to XIAP, can thus block the function of inhibitors of apoptosis, that is why the use of AT-406 or other Smac mimetics provides an opportunity to reactivate caspase 9 [63]. Altogether, the above-stated data highlight the importance and topicality of further careful investigation of caspases and endogenous factors governing their activity in the context of the problem of cervical cancer diagnostics and treatment.

2.4.2. Expression and activity of caspases and CD95 level in PBMC from patients with CIN 3 and CC

The expression of either HPV-specific or tumor-associated antigens by neoplastic CC cells and exposure of these antigens to the immune system eventually drive to the activation of specific lymphocyte subpopulations. At the same time, the activation of immunocompetent cells can proceed in parallel with induction of apoptosis that may predetermine, at least partially, the inefficacy of antiviral/antitumor reactions of the immune system. Although there is some published data showing the upregulation of apoptosis in lymphocytes infiltrating cervical cancer tissue [27, 28], the following questions remain under investigation: To what extent apoptosis-associated processes are present in systemic circulation of CC patients? What signaling pathways might be involved in this case? Could the apoptosis-related changes be detected in peripheral blood of patients with preinvasive cancer? Within the task of testing assumption that the mechanisms governing the apoptotic program do exacerbate in the circulating lymphocytes upon CC development, especially with regard to its early stages, we analyzed the level of protease activity of receptor-regulated caspase 8, executioner caspases 3 and 6, and caspase 9 initiating the intrinsic apoptotic pathway, in the mononuclear fraction of blood samples, taken from women with CIN 3 or CC, in comparison with the group of healthy controls (Figure 4).

In 50% of CIN 3 cases, the activity of caspase 8 matched the control level, whereas in the rest 50%, it was notably increased. In invasive cancer, the activity of caspase 8 was significantly higher (in 100% of samples) than the control level, showing strong positive correlation with the stage of the disease ($r = 0.92$, $R^2 = 0.86$, $p < 0.01$) (Figure 4A). Similar correlation was observed for caspase 6: in 100% of invasive CC patients, its activity was significantly (4–5 times) higher relative to the control group ($r = 0.77$, $R^2 = 0.59$, $p < 0.01$); however, unlike caspase 8, in most of CIN 3 cases (77%), the activity of caspase 6 corresponded to the control values (Figure 4B). The relationship between the level of caspase 3 activity and the stage of cancer occurred to be

nonlinear: upon CIN 3 → stage IA progression, the caspase 3 activity increased, while with stage II → IV progression, it gradually diminished up to the control levels, in 5 cases falling to almost undetectable levels (R^2 = 0.45, p < 0.01, polynomial regression of II order). It is noteworthy that for all examined patient groups, there was a certain percent of samples (35% of CIN 3, 30% of CC stage IA, 38% of CC stage II), with caspase 3 activity showing no difference as compared to the control (Figure 4C). In contrast to caspases 3, 6, and 8, caspase 9 exhibited reduced activity as the stage progressed (r = –0.60, R^2 = 0.36, p < 0.01); however, in 26% of stage IA blood samples, elevated caspase 9 activity was detected. In CIN 3 group, reduced caspase 9 activity was revealed for 40% of samples (Figure 4D). We also examined whether these caspase-specific changes observed at the level of enzymatic activity could extend to the transcriptional level. The correlation of the relative mRNA expression levels of caspases 3 and 6 with the cancer stage displayed, in general, the same character as the activity level, being, however, much less pronounced (data not shown). As for caspase 9 mRNA level, it was not found to be correlated with the stage of CC.

Summarizing the findings stated above, we can conclude that CC progression is associated with the specific change of activity pattern of caspases 8, 3, and 6 that are united by being components of extrinsic, receptor-mediated pathway of apoptosis. Importantly, the systemic fluctuations of caspase activity revealed in the circulating PBMC appear to be an early event in CC development—upregulation of all three caspases were already detectable at the stage of microinvasion for the most of samples, and for substantial portion of samples—at the stage of intraepithelial cancer (CIN 3). It is obvious that molecular factors and mechanisms by which HPV or a developing neoplasia can exert systemic influence on the immune system still remain largely unknown and define trends of future research, but nevertheless there is growing body of evidence that the development of a malignant process can raise considerable changes in gene expression profile of peripheral blood leukocytes, with certain fraction of genes being related to apoptosis signal transduction and implementation of the cell suicide program [64–66]. The upregulation of caspases 8, 3, and 6 activity observed in our study may represent a direct consequence of increased membrane expression of cell death receptors—CD95/APO-1/Fas first of all as the key acceptor of apoptotic signals on the surface of lymphocytes. An increase in the number of CD95-expressing peripheral blood lymphocytes was revealed for patients with hepatocarcinoma [67], melanoma [68], ovarian cancer [69], head and neck cancer [70], gastric [71], nonsmall cell lung cancer [72]. That is why we decided to examine if a similar phenotypic change of circulating lymphocytes could occur along with CC progression.

By using flow cytometric assay, we explored the level of surface expression of CD95-marker in the blood lymphocytes of CIN 3 and CC patients in comparison with the control group. As follows from Figure 5, the number of CD95-expressing cells in CIN 3 group was higher than that of control, exhibiting further increase with CC stage progression. Together with our data on activity of caspases, these results allow us to assume that circulating lymphocytes become more susceptible to Fas-mediated apoptosis. It is worth mentioning, however, that CD95 represents a marker with "dual" functionality: performing a function of a cell death receptor, CD95 serves at the same time as an early activation marker of T-lymphocytes [73]. Taking into account the fact that cervical neoplastic lesions develop on the ground of chronic HPV infection, one can connect the observed elevation of CD95 to the processes of activation of the T cell-mediated branch of immunity, induced by the sustained expression of viral antigens

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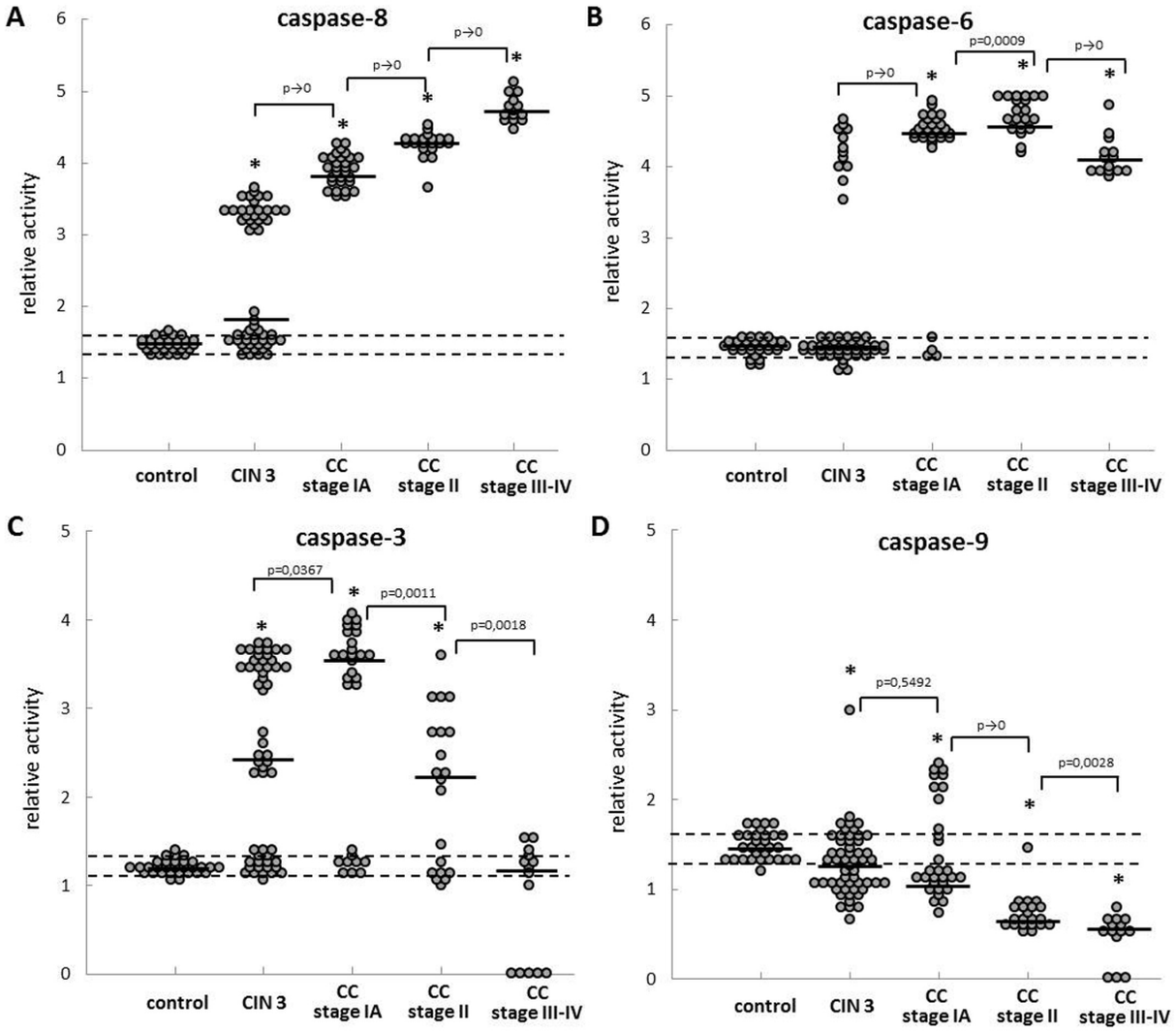

Figure 4. Scatter plots showing individual patients' changes of protease activity of caspases 3(A), 6(B), 8(C), and 9 (D) in PBMC at different stages of CC development. Horizontal bars represent the median activity level for each data set; dashed lines indicate ± standard deviation (SD) of the mean value in samples of the control group. The results were multiplied by 10^4 for convenience. *Patient groups with median caspase activity being significantly different from that of control ($p < 0.05$). The p-values shown on the plots were obtained by comparing successive stages of CC.

and their exposure to the antigen-presenting cells. Considering this potential cause, we narrowed the analyzed population by adding a pan T-cell marker (CD3) and determined the number of lymphocytes having $CD3^+CD95^+$ or $CD3^+CD95^{high}$ phenotype in the blood of CIN 3 patients, as compared with the control group (Figure 5). A significant increase in the percentage of $CD3^+CD95^{+/high}$ T-cells regarded as effector T-lymphocytes was found in CIN 3 women. Furthermore, we have seen that this change of CD95 expression affected primarily the subpopulation of T-helpers ($CD3^+CD4^+CD95^+$ and $CD3^+CD4^+CD95^{high}$ phenotypes) (Figure 5) but was not characteristic of cytotoxic T-cells (defined by $CD3^+CD8^+CD95^{+/high}$ phenotype), thus supporting the results of other studies disclosing the dominant role of the T-helper branch of immunity in the processes of CC carcinogenesis (reviewed in [74, 75]).

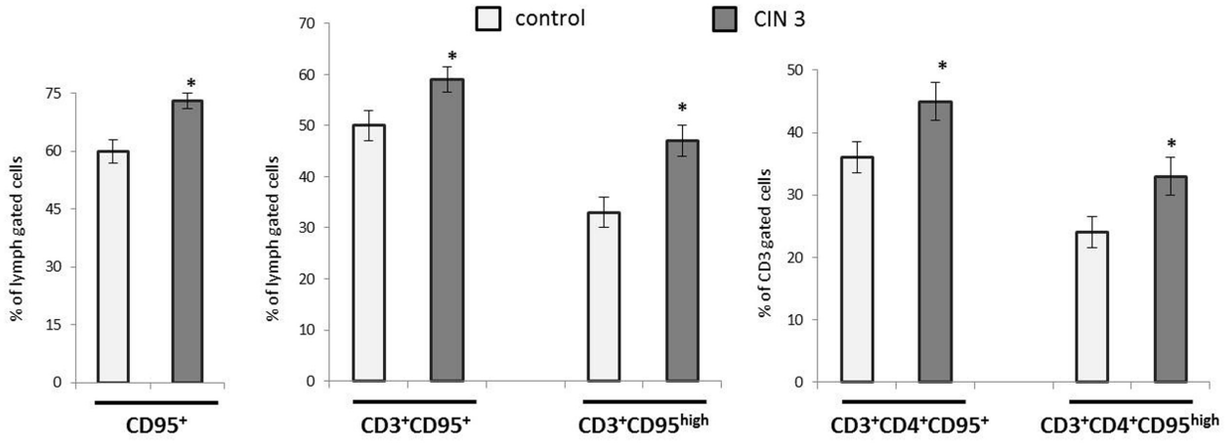

Figure 5. Expression of CD markers in peripheral blood lymphocytes of CIN 3 patients. *$p < 0.05$ (U-test).

Does the intensification of the apoptotic death of circulating leukocytes take place in the blood of women suffering from CIN or cervical cancer, and, if so, under the influence of what humoral factors does it occur? Which of the cell subpopulations becomes the most susceptible one? What is the impact of apoptotic processes on immune status and disease progression? To answer these questions, further research is undoubtedly needed to be done. Of special interest are those immune state disorders that support the persistence of HPV infection and promote transition of the disease from intraepithelial form into true cancer and microcarcinoma. At the current step of our research, additional corroboration of potentiation of apoptosis-related reactions in the blood lymphocytes upon CC development comes from observation that in the course of treatment, the immune status recovery process was accompanied by normalization of the level of CD95-marker expression and activity of caspases 8, 3, and 6.

2.4.3. Activity of caspases and CD95 level in PBMC of patients with CIN 3 and CC after treatment

At present, new approaches to immunotherapy/immunomodulatory therapy for the HPV infection and cervical neoplasms designed for the reduction of the HPV clearance period, prevention of reinfection, and activation of mechanisms of nonspecific immune defense are being intensively developed (for a review, see, for example [76, 77]). However, the problem of putting such approaches into practice is closely related to the task of finding adequate criteria for assessment of their clinical effectiveness. Besides, regardless of treatment strategy (standard, surgical, or combined, including immunomodulatory therapy), there exists a need to monitor the recovery of a patient's immune status during the postoperative period and to estimate the individual response to therapy, which, in turn, requires an appropriate set of biomarkers. Considering these points, we assessed the change of caspase activity level and CD95 expression in circulating PBMC of women diagnosed with CIN 3 or microinvasive CC (stage IA1) after treatment. Repeated blood samplings were done in 1 and 3 months after diathermoconization and the course of immunomodulatory therapy. The control group 2 was consisted of patients with CIN 3 (n = 15) and CC of IA stage (n = 15), who had received only surgical treatment in full accordance with the approved standard. As it follows from Figure 6, in the blood lymphocyte fraction of patients constituting the control group 2, the expression

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of CD95 marker did not alter significantly within the 3-month period, whereas immunomodulatory treatment elicited marked reduction in the number of CD95-positive cells. It is worth to mention that the more noticeable changes of CD95 expression in the group of receiving complex treatment were associated with the more effective elimination of HPV infection.

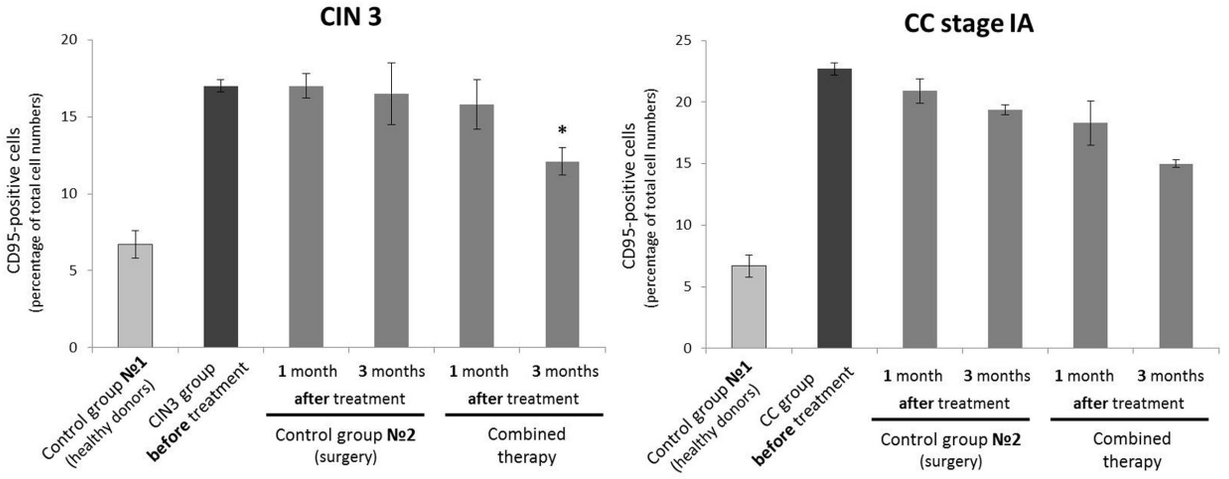

Figure 6. Change of CD95 expression in peripheral blood lymphocytes of patients diagnosed with CIN 3 or microcarcinoma of the cervix after treatment. Mean values ± SD are shown. *Significant difference between the control group 2 (3 months after treatment) vs. CIN3 group of patients received combined therapy ($p < 0.05$, *U*-test).

Resting on the obtained results, indicating gradual normalization of the numbers of circulating CD95-positive lymphocytes after the course of treatment, we decided to explore if this change could be coupled with normalization of the activity/expression levels of caspases. After treatment, the change of caspase 8 activity was most pronounced (Figure 7A): in CIN 3 and CC samples, it appeared to return to the control levels after 1 or 3 months, respectively. Only 15% of CIN 3 samples still exhibited the increased activity of caspase 8 after treatment, as compared with 50% before treatment. The activity of caspase 6 (Figure 7B), as opposed to caspase 8, was elevated in 60% of the CIN 3 cases after treatment (although before treatment it was upregulated only in 23% of cases). In CC stage IA group after treatment, caspase 6 activity was revealed to correspond to the control levels for the most of samples tested; however, elevated activity was also detected in several cases. The pattern of caspase 3 activity (Figure 7C) measured in PBMC of patients with CIN 3 and CC stage IA after the treatment course remained actually unchanged, with a slight (statistically nonsignificant) decrease in the median values in each group. In CIN 3 group, both before and after treatment, increased caspase 3 activity levels were observed in 60% and 45% of PBMC samples, respectively. As was stated before, in contrast to caspases 8, 3, and 6, the activity of caspase 9 was shown to diminish with the disease progression (including CIN 3 and CC stage IA). After treatment, caspase 9 activity was shown to match the control level or to be increased (Figure 7D). Interestingly, if before treatment there were no CIN 3 cases with elevated caspase 9 activity (except for a single case), 7 of PBMC samples demonstrated caspase 9 upregulation after the course of treatment. Although it was the level of proteolytic activity of caspases, but not mRNA levels, that displayed the most significant change in CIN 3 and CC stage IA samples, we

nevertheless have analyzed mRNA expression of caspases in PBMC samples of these patient groups after treatment but did not find any significant differences (data not shown).

Altogether, the results obtained indicate that after the course of treatment, a restoration of caspase activity level up to the control values does occur in the blood lymphocytes of CIN/CC patients, with the maximum effect observed for the initiator caspases 8 and 9. By this, we confirm the specificity of changes of the selected indicators, namely, the activity profile of the initiator and executioner caspases, establish their relevance to neoplastic progression, and reinforce the possibility of using caspases as biomarkers of the early steps of cervical cancer development.

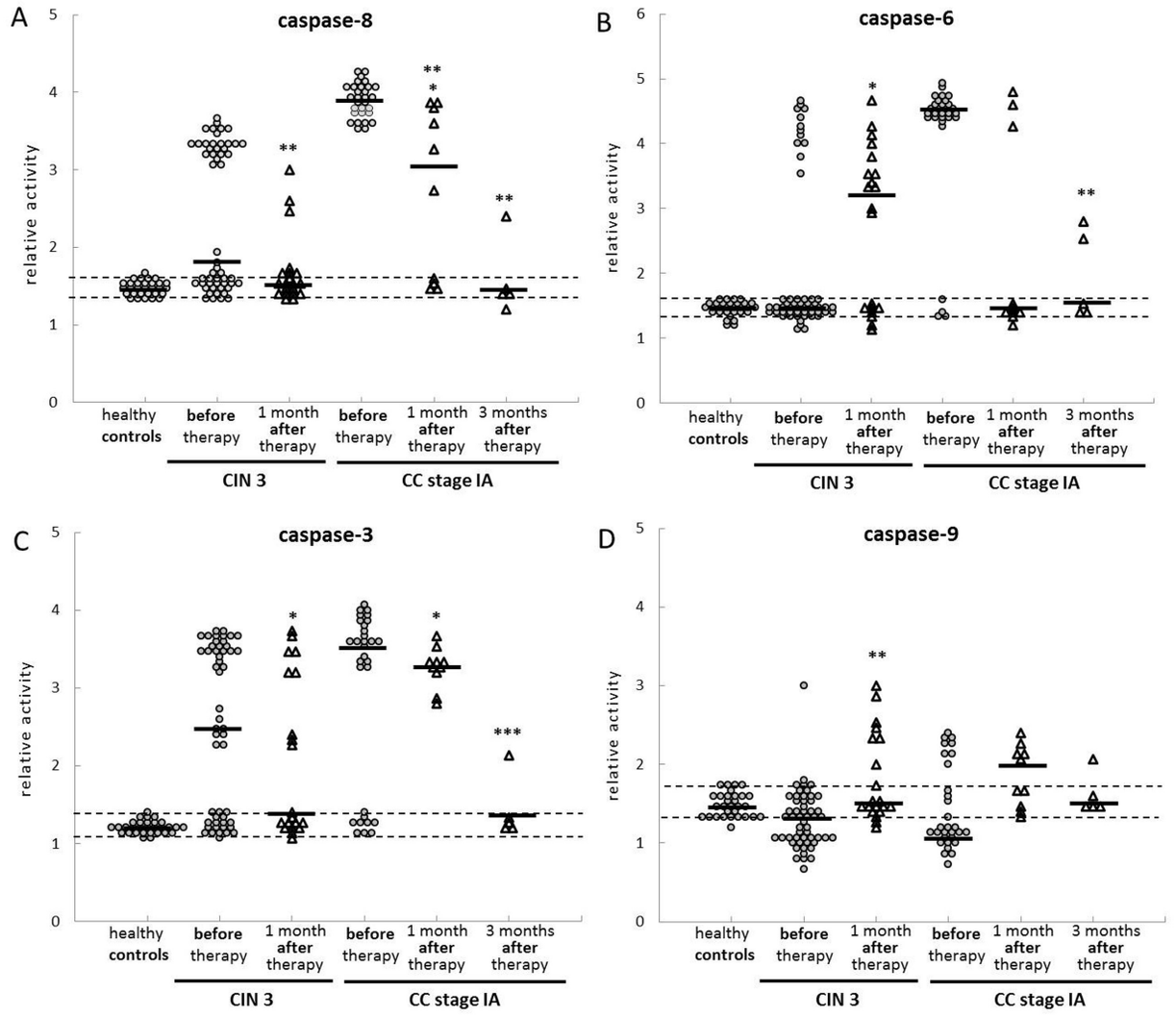

Figure 7. Scatter plots showing individual patients' changes of protease activity of caspases 8 (A), 6 (B), 3 (C), and 9 (D) in PBMC of CIN 3 and CC stage IA groups as measured before and after treatment. Horizontal bars represent the median activity level for each data set; dashed lines indicate ± standard deviation (SD) of the mean value in samples of the control group. The differences were considered significant if *p*-value was <0.01 (*U*-test). *Significant difference between the patients and the healthy controls. **Significant difference between caspase activity levels measured before vs. after treatment within the same group. ***Significant difference between 1- and 3-month periods in a group of patients diagnosed with stage IA.

3. Conclusions

By the present study, we intended to show that caspases are pivotal components of the molecular "portrait" of CIN and cervical cancer characterizing the processes of establishing a basic phenotypic trait of a tumor—resistance to apoptosis-inducing factors. By measuring the activity of caspases, we were able to differentiate the early steps of CC progression (intraepithelial and microinvasive cancer) and reveal systemic deviations in the cellular branch of immunity. We suppose that the development of the ability to suppress apoptotic death evaluated by the level of caspase activity can serve as a pointer to malignant transformation of CIN, to the readiness of epithelial cells to counteract the immune reactions, and may predispose to further disease progression. The data accumulated to date provide grounds for considering caspases as promising biomarkers for comprehensive diagnostics of CIN and early forms of CC (in combination with other molecular markers of the viral or cellular origin, as well as with morphological criteria). Nevertheless, it should be noted that more research is required for successful translation of the described markers into clinical practice.

In addition to the prospects of clinical application, the results obtained define possible directions of further fundamental research as, for example, the investigation of molecular factors and mechanisms controlling induction of apoptosis-related processes in immune cells of HPV/CIN-positive individuals, as well as the mechanisms regulating caspase activity in neoplastic cells of the cervix, the elucidation of the impact of apoptotic pathways on systemic immune suppression upon the development of oncopathology. No doubt that the model of regulatory relationships that provide the abrogation of apoptosis in CC cells or its induction in lymphocytes still contain a lot of "gaps" and that is why a great many of the current studies are aimed at identification of the novel molecular players of the apoptotic signaling pathway functionally linked to caspases. For example, it emerges from these studies that TWEAK/Fn14- and TRAIL-mediated pathways of triggering cell death are deeply integrated into the mechanisms of CC development [4, 78]. At the same time, more and more attention is being given to the exploration of alternative variants of PCD and their cross-talk with the mechanism of caspase-dependent apoptosis, for example, there is some new data giving insight into the role of autophagy and endoplasmic reticulum (ER) stress-induced cell death in CC carcinogenesis [79]. Caspases are supposed to function as molecular "switches" between different pathways of programmed death—autophagy and apoptosis—under the influence of different types of treatment on cervical cancer cells [80–82]. These facts emphasize one more time the universality of caspases and stress their importance for studying various aspects of the problem of cervical cancer development and treatment.

4. Appendices and nomenclatures

CC—cervical cancer, CIN—cervical intraepithelial neoplasia, DISC—death-inducing signaling complex, HPV—human papillomavirus, IAPs—inhibitors of apoptosis protein family, PBMC—peripheral blood mononuclear cells, PCD—programmed cell death.

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