ANALYSIS OF EXPRESSION OF AIF AND PARP-1 IN CANCERGENESIS OF HPV-POSITIVE CERVIX

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Abstract

The mRNA expression of AIF and PARP-1 in HPV negative non-tumour epithelial cells and HPV-positive cervical cancer cells using the real-time PCR method was examined. An increased level of AIF and PARP-1 mRNA in cervical cancer cells in comparison to normal epithelial cells was demonstrated. These results suggest that changes in the mRNA expression level of AIF and PARP-1 might be involved in cervical cancer development. The analysis of these two factors may represent a new molecular tool for cervical cancer prevention in women with HPV persistent infection.

Key words: women, human papillomaviruses, cervical cancer, PARP-1, AIF.

Human papillomaviruses (HPVs), especially types 16 and 18, are the main agent of cervical carcinogenesis. Two early viral proteins E6 and E7 are essential for the tumour transformation properties of high risk HPVs. These proteins interact with the tumour suppressor proteins p53 and RB respectively to inhibit apoptosis (11, 12). The E6 viral protein of HPV16 and HPV18 interacts and promotes inactivation of other cellular proteins that are involved in cell proliferation and apoptosis, such as Bak or Myc. Early proteins of HPV might also affect the release of cytochrome c and AIF from mitochondria (5).

HPV mediated transformation of epithelial cervix cells is a multi-step process in which not only oncogenic viruses but also additional, yet unknown, genetic and epigenetic factors are required. It is believed that some mitochondrial and cell death factors as AIF (apoptosis-inducing factor) and PARP-1 [poly (ADP-ribose) polymerase 1] may play an important role in this process.

PARP-1 is an abundant nuclear enzyme, encoded by the ADPRT (ADP-ribosyl transferase gene), involved in DNA-repair and modification of proteins. This protein belongs to the PARP family of proteins that includes also PARP-2, PARP-3, and tankyrases 1 and 2, and is the best known member of the PARP family. PARP-1 activity rapidly increases in response to DNA damage in a cell. Therefore, PARP-1 can also regulate replication of DNA, transcription, cellular differentiation, cell death, and malignant transformation (1, 4). PARP-1 is emerging as an important activator of caspase-independent cell death (8, 9).

PARP-1 is a 116 kDa protein that consists of three functional domains: N-terminal domain (42 kDa) that is a DNA-binding domain and contains a nuclear localisation signal (NLS), C-terminal catalytic domain (55kDa), and a central automodification domain that is placed between N- and C-terminal domains. Two zinc-finger motifs in the N-terminal domain of PARP-1 are responsible for recognising breaks in double-stranded DNA (dsDNA). PARP-1 is a highly conserved protein in structural and functional organisation.

Under normal physiological conditions, PARP-1 activity is important for regulation of cellular homeostasis and maintenance of genomic stability. It has pro-survival functions in terms of DNA-repair, thus its activity in cancer is highly increased, and it is one mechanism through which cancer cells avoid apoptosis (1, 8).

AIF is a mitochondrial protein, which is located in the mitochondrial intermembrane space-anchored in the inner mitochondrial membrane. AIF is indispensable for the functioning of the mitochondrial respiratory chain. It is released from mitochondria to the cytosol during mitochondrial outer membrane permeabilisation (MOMP) and then is translocated to the nucleus. There AIF is responsible for chromatin condensation and DNA degradation (large scale ~ 50 kb DNA fragmentation). Apoptotic release of the membrane-anchored AIF requires its cleavage. It is known that calpain I and/or cathepsin provokes the cleavage and release of AIF from the mitochondria. Nonetheless, it is now supposed that the accumulation of PAR (poly (ADP-ribose) polymers substrates of PARP-1 mentioned before, may cause the release of AIF from the mitochondria in the absence of AIF cleavage by calpain I. The molecular connections between PARP-1 and AIF in cell death still remain elusive and under investigation (4, 7, 8).

The mRNA of AIF is subjected to alternative splicing. Precursor of AIF is a 67 kDa protein that
contains MLS (mitochondria localisation signal), IMSS (inner membrane sorting signal), FAD-binding domain, NADH-binding domain, NLS (nucleous localisation signal), and the C-terminal domain. After being compartmentalised in mitochondria, AIF is processed, MLS is removed, and the mature form of protein is 57 kDa. The C-terminal domain seems to be the pro-apoptotic part of AIF (4).

Recent studies have indicated that AIF also has a pro-survival function. Due to the fact that AIF bears NADH-binding domain and two FAD-binding segments, it may act as a mitochondrial oxidoreductase and play a role in oxidative phosphorylation. Recently, it has been shown that AIF regulates the respiratory chain complex I, may be implicated in stabilisation of complex I subunits, acts as a free radical scavenger, and protects cells from cell death induced by oxidative stress (13, 15). Thus, AIF is a bifunctional protein, which plays a major role in cell death and on the other side, as NADH oxidase plays a role in oxidative phosphorylation. The relation between the oxidoreductase activity of AIF, oxidative phosphorylation, complex I, and cell survival remains to be established.

The aim of this study was to estimate the mRNA (transcript) level of AIF and PARP-1 in tissues from HPV-negative and HPV-positive women, who suffer from low-grade squamous epithelial lesion (LSIL), high-grade squamous epithelial lesion (HSIL), and cervical cancer, using real-time reverse transcriptase-polymerase chain reaction (RT-PCR). RT-PCR method was also used to evaluate the expression level of early HPV’s protein (E6, E7) in HPV positive material.

Material and Methods

Postoperative cervical cancer tissues, dysplastic tissue, and normal, non-tumour cervical tissue used as a control, were examined. These samples consisted of eight carcinoma colli uteri/squamous cell carcinoma HPV-positive, 31 H-SIL (high-grade squamous epithelial lesion) HPV- positive, 20 L-SIL (low-grade squamous epithelial lesion) HPV-positive, and 16 histopathologically normal, HPV-negative cervical tissues obtained from women undergoing treatment for reasons other than cervical cancer. The material was obtained from women, who had been treated at the Department of Obstetrics and Pathology of Pregnancy, Medical University, Lublin. Samples containing at least 70% tumour cells were used. The samples were put in RNAlater RNA Stabilization Reagent (Qiagen) as soon as possible and stored at -80°C until use.

RNA extraction/isolation. Total RNA was isolated from normal, dysplastic (L-SIL and H-SIL), and cancer tissues with RNase Mini Kit (Qiagen) according to the manufacturer’s instructions. DNA was removed by DNase treatment (RNase-Free Dnase Set, Qiagen).

RT-PCR analysis. Total RNA was reverse transcribed to cDNA using the QuantiTect Reverse Transcription Kit (Qiagen). cDNA was prepared from 1 μg RNA template in 28 μl reaction mixtures. Real-time PCR was performed on a Real-time machine, Corbett Rotor-Gene 6000, using SYBR Green PCR Master Mix (Applied Biosystems, U.K.), and appropriate primers for AIF and PARP-1 genes. Two microlitres of total cDNA was added to 8 μl of Power SYBR Green PCR Master Mix (Applied Biosystems) and primers. AIF-1 isoform was amplified using primers as described previously (17). The expression of AIF and PARP-1 was normalised by comparison with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and large ribosomal protein PO (RPLPO) expression. GAPDH and RPLPO were amplified with specific primers as described earlier (3). For normalisation of the AIF and PARP-1 expression, the geNorm programme (version 3.4) was used. All reactions for samples and housekeeping genes were run in triplicates. The primers used for amplifying AIF, PARP-1, GAPDH, and RPLPO are listed in Table 1.

Statistical analysis. The expression level of AIF and PARP-1 was statistically evaluated by calculating the mean value for control, LSIL, HSIL, and cancer samples. The confidence interval was done for all samples with a 0.05 significance level. Furthermore, to confirm that the expression level of AIF and PARP-1 mRNA is higher in cancer tissues than in control samples, two statistical hypothesis tests were done using Student’s t-test for two samples with unequal variance. In these tests, a P-value 0.05 for PARP-1 and 0.12 for AIF were received. If the standard P value 0.05 is adopted, then the received higher value of expression of PARP-1 mRNA in cancer samples in relation to the control samples can be accepted as statistically significant unlike the higher value of expression of AIF mRNA, which should be accepted as statistically insignificant. A high P value = 0.12 for AIF is a result of using only a few cancer samples in the statistical analysis. We noticed that the expression of PARP-1 mRNA positively correlates with the expression of AIF mRNA in cancer samples – Pearson’s correlation coefficient r = 0.82. The results are presented in graphs (Figs 1 and 2).

<table>
<thead>
<tr>
<th>Primer sequences used for RT-PCR analysis</th>
<th>Primers sequence (5'→ 3')</th>
<th>References</th>
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<tbody>
<tr>
<td>AIF</td>
<td>sense GATTGCAACAGGGAGGTAGTCTCCCAAGA antisense GATTGTGACTTCCCCCTAAATCCTTC</td>
<td>(17)</td>
</tr>
<tr>
<td>PARP-1</td>
<td>sense GATTGCAACAGGGAGGTAGTCTCCCAAGA antisense GATTGTGACTTCCCCCTAAATCCTTC</td>
<td>The primers designed using the Primer - BLAST (BLAST/ NCBI)</td>
</tr>
<tr>
<td>GAPDH</td>
<td>sense AAGGACGGCAGGTCTCACGATTT antisense ACCAGATTTAAAGGACGCTCTTGG</td>
<td>(3)</td>
</tr>
<tr>
<td>RPLPO</td>
<td>sense CCTCATAATCCGGGGGGATGTTG antisense GCAGCAGGTCGGCCTTTAT</td>
<td>(3)</td>
</tr>
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References

(3) The primers designed using the Primer - BLAST (BLAST/ NCBI)
Results

The total RNA was isolated from precancerous, cancerous, and control tissues and was used for the identification of HPV DNA (Table 1). The same clinical samples were used for RNA isolation and analysis of mRNA expression of PARP-1 and AIF using Real-time PCR. Figs 1 and 2 illustrate the mRNA expression of PARP-1 and AIF in precancerous and cancerous tissues, respectively.

The RT-PCR analysis revealed a high expression of AIF and PARP-1 in cervical cancer samples on the mRNA level. An increased expression of HPV16 viral oncoproteins E6 and E7 on the mRNA level in HPV positive cancer samples was also observed (Figs 3 and 4).

Discussion

It has been claimed for many years that the deregulation of survival and death of cells plays an important role in cancerogenesis. We focused on two proteins AIF and PARP-1, because they play a significant role in apoptosis. Some studies have demonstrated that AIF gene expression could be changed in cancer cells (7). In our study we evaluated the expression of AIF and PARP-1 in cervical cancer HPV-positive cells using the RT-PCR method. Increased expression of AIF in cancer cervical cells compared to the normal cervical tissue suggests that AIF expression might play a role in cervical tumourgenesis. AIF is a pro-apoptotic protein, but also has the NADH oxidase activity, and plays a function of ROS scavenger, thus can protect cells from cell death (8, 10, 20).

We also observed an increased level of PARP-1 in cancer tissues. It is known that the increased activity of PARP is one mechanism by which cancer cells can avoid apoptosis and can survive (9). The cell-death pathway initiated by PARP-1 activation appears to be mediated by AIF (7). Since PARP-1 is activated in response to DNA damage in cells, studies have been conducted to find inhibitors of PARP-1. Inhibition of PARP-1 prevents the repair of DNA breaks enabling cells to undergo apoptosis process.

We also investigated the absence of viral E6 and E7 proteins in cervical cancer HPV positive samples (Figs. 3 and 4). High-risk HPV E6 protein has the ability to interact with critical cell regulatory protein p53 and leads to its functional inactivation (11, 12, 19). The p53 plays a pivotal role in suppressing tumourigenesis by inducing genomic stability, cell cycle arrest, or apoptosis. AIF gene expression is positively regulated by p53. The induction of AIF expression by p53 plays a dual role: AIF is a mediator of cell death following from the mitochondria to the nucleus and has an important cytoprotective function, defending the cell against oxidative damage and other types of stress (17).

As a result of neutralisation of the p53 function, a fundamental cellular event in the cell cycle, apoptosis and genomic stability is disrupted in HPV-high risk positive cells.
DNA damage in cancerous cells can also lead to an increased mRNA level of PARP-1 (18). The destabilisation of p53 and the high expression level of PARP-1 in cervical cancer tissues suggest that cancerous cells may not effectuate the apoptosis process and consequently the tumourigenesis process is initiated in the cervix. PARP-1 plays a key role in caspase-independent cell death and mediates the release of AIF from the mitochondria. Interestingly, in our study Realtime PCR analysis showed also an increased level of AIF transcript in cervical cancer samples. It may suggest that over-expression of AIF appears in response to the genomic instability in cells. On the other hand, AIF has a dual role in the cell as described above. It can protect cells against the consequences of oxidative damage and has a pro-survival function. The presence of E6 and E7 HPV-high risk oncoproteins and increased mRNA expression of PARP-1 indicates that HPV infected cells cannot undergo the effective apoptosis process despite increased mRNA levels of AIF in cervical cancer tissues. However, further analysis is required to clarify the correlation between viral infection, an increase in mRNA expression levels of PARP-1 and AIF, inhibition of the apoptosis process, and tumourigenesis in the cervix cells.

In summary, a better understanding of the mechanism of the mitochondrial factor AIF, and PARP-1 might lead to the development of new therapies for many diseases, which are associated with DNA damage, mitochondrial dysfunction and disorder of cell death pathways. Nowadays, the development of successful inhibitors of PARP-1 can be an effective tool for the treatment of cancer diseases (2, 6, 9, 14, 16). The presented data supplement the knowledge of AIF and PARP-1 in regard to cervical cancer and help to better understand the mechanisms that regulate life and death in cells.

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References