REVIEW

'Drawing' a Molecular Portrait of CIN and Cervical Cancer: a Review of Genome-Wide Molecular Profiling Data

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Abstract

In this review we summarize the results of studies employing high-throughput methods of profiling of HPV-associated cervical intraepithelial neoplasia (CIN) and squamous cell cervical cancers at key intracellular regulatory levels to demonstrate the unique identity of the landscape of molecular changes underlying this oncopathology, and to show how these changes are related to the 'natural history' of cervical cancer progression and the formation of clinically significant properties of tumors. A step-wise character of cervical cancer progression is a morphologically well-described fact and, as evidenced by genome-wide screenings, it is indeed the consistent change of the molecular profiles of HPV-infected epithelial cells through which they progressively acquire the phenotypic hallmarks of cancerous cells. In this sense, CIN/cervical cancer is a unique model for studying the driving forces and mechanisms of carcinogenesis. Recent research has allowed definition of the whole-genome spectrum of both random and regular molecular alterations, as well as changes either common to processes of carcinogenesis or specific for cervical cancer. Despite the existence of questions that are still to be investigated, these findings are of great value for the future development of approaches for the diagnostics and treatment of cervical neoplasms.

Keywords: Cervical cancer-CIN-HPV-molecular profile-genome-signature

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Introduction

Owing to contemporary high-throughput techniques for biomedical data sampling and analysis, any state of the cell-from physiological '*norm*' to pathology-can be described as a set of molecular profiles (genome, transcriptome, epigenome, proteome, metabolome) that constitute its overall molecular-genetic '*portrait*'. By comparing these cellular '*portraits*' one can ascertain the presence of a pathological process, unambiguously classify it, determine the lesion causes and grade, identify case-specific features. Cancer is a complex highly heterogeneous disease, usually progressing over a prolonged time period through accumulation of multiple genetic and phenotypic disorders at all levels of intracellular regulation. That is apparently why the molecular '*portrait*' concept is primarily associated with this type of pathology. Since the background for tumor formation and growth is continuous mutagenesis, microevolution and changes in the tissue micro-environment, its molecular-genetic '*portrait*' is dynamic and tightly bound to the stage of the disease.

Cervical cancer (CeCa) is an example of oncopathology, for which the whole continuum of carcinogenesis, from the earliest stages-cervical intraepithelial neoplasia (CIN) of grades 1, 2, 3, as well as microinvasive cancer, have been described in sufficient detail relying on morphological criteria. In this sense, CeCa is a unique *in vivo* model for studying the driving forces and mechanisms of carcinogenesis. As indicated by research on genome-wide screening of molecular abnormalities in CIN/CeCa bioptates, it is indeed the consistent change of the molecular profiles of epithelial cells that underlies the observed morphological changes. Different phases in CIN and CeCa progression have their specific '*portraits*', and analysis of such '*portraits*' is a way to identify the mechanisms behind the malignant transformation and progression of the tumor under natural conditions.

In an overwhelming majority of cases, CeCa development is linked to a '*high-risk*' (mainly of types 16 & 18) human papillomavirus (HPV), which is regarded as the primary inducer of all subsequent changes in the molecular-genetic '*portrait*' of the infected cells in the epithelium of the cervix. The functioning of the viral genome and the principles of HPV life cycle regulation have been intensively investigated using various experimental systems (Doorbar et al., 2012). It is common knowledge that a number of virus-specific proteins-E5, E6 and E7-possess oncogenic properties. In the productive life cycle, the virus oncoproteins keep the cellular

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replication system constantly active, so that infected cells on their terminal differentiation pathway retain the capacity to initiate the S phase (Ibeanu, 2011). This leads to a morphologically apparent hyperplasia of cervical epithelium in the locus of infection (CIN1). As epithelial cells differentiate further, E6 and E7 pro-proliferative activity is counterbalanced by the mechanisms for negative regulation of the cell cycle, both on the part of the host cell and on the part of the virus (Doorbar, 2006). Collectively, these processes facilitate long-term persistence of the virus without inducing malignant growth. It is commonly held that the triggering mechanism for neoplasia progression (CIN2/3) is a loss of control over the level of E6 and E7 expression. It had long been believed that the reason for that was spontaneous HPV integration into the host cell genome. Nowadays however, alternative mechanisms for E6 and E7 deregulation are being postulated, suggesting that the virus transition from the extrachromosomal to the integrated state is a side effect of the oncotransformation rather than its trigger (Ibeanu, 2011).

Recent research findings show that the essence of HPV-induced transformation is far more complex that it is generally thought. E6- and E7-dependent degradation of key regulators of the cell cycle and tumor suppressors p53 and pRb is still believed to be most critical factor. Yet, studies of interactomes, i.e. the set of interactions between viral proteins and host cell proteins, revealed numerous direct targets of E6 and E7 oncoproteins, in addition to p53 and pRb. These targets include proteins that control cell morphology, cell polarity and intercellular adhesion, various transcription factors and transcriptional coactivators, intracellular proteolysis and intracellular traffic regulators, chromatin remodeling enzymes, tumor suppressors, protein kinases, and protein phosphatases (Halim et al., 2013). Of principal significance is HPV-oncogenes mediated activation of telomerase, which is essential for maintaining immortalized state of CIN and CeCa cells (Petrenko et al., 2010; Zhao et al., 2015). *In silico* analysis also revealed entire cell signaling pathways targeted by HPV oncogenes, namely Wnt-, Akt-, Notch-, mTORC-, STAT-dependent signaling cascades (Doorbar et al., 2012). Thus, it is safe to say that the transforming effect of HPV rests upon total reprogramming of basic cell functions (proliferation, differentiation, apoptosis, maintaining invariable genome structure), i.e. modification of the molecular '*portrait*' of the target cell. It appears however that the virus oncogenes perform only the initiating function, as they cannot *per se* cause oncotransformation. The oncogenic potential of the virus is unraveled through stimulation of genomic instability and gradual accumulation of somatic mutations affecting cell proto-oncogene functioning.

In this review we summarize the results of studies employing methods of profiling of HPV-associated CIN and squamous cell CeCa at the genome, epigenome, transcriptome, and proteome levels to demonstrate the unique identity of changes in the molecular-genetic '*portrait*' of this oncopathology, and to show how these changes are related to the 'natural history' of CeCa progression and the formation of clinically significant properties of the tumor. Although CeCa research has advanced considerably, one must say that finding correlations between changes at the morphological/phenotype and the molecular levels is still a fundamental challenge. To wit, it still remains unknown which molecular phenotype (the set of molecular-genetic aberrations) of epithelial cells is associated with the irreversible stage of neoplasia progression and acquisition of the invasion capacity. Neither do we know why CIN regresses spontaneously in a majority of cases and only a minor fraction progresses to CeCa, or which processes are the driving forces of the regression/progression.

Chromosomal Aberration Profile

A distinctive feature of CeCa molecular-genetic '*portrait*' is a high degree of genome instability that forms at the earliest stages of the disease (CIN2/3) and is regarded as the immediate cause of the malignant transformation. At the same time, no driver point mutations have been found for CeCa (Narayan and Murty, 2010), in contrast, for instance, to breast cancer, where genome-wide instability is secondary to specific gene mutations (*brca 1/2, ras, b-raf, etc*.).

Genome destabilization is a direct consequence of the activity of HPV oncogenic proteins. On the one hand, by stimulating the proliferation of infected cells, E6 and E7 eliminate numerous cell cycle '*checkpoints*', thus promoting gradual accretion of somatic mutations; on the other hand, HPV oncogenes can cause gross chromosomal rearrangements as they affect the centriole duplication mechanism, thus altering the mitotic spindle polarity and the daughter chromosome segregation process (Doorbar et al., 2012). Another source of DNA structural damage is an increased formation of reactive oxygen species and a reduced activity of superoxide dismutase and glutathione peroxidase due to expression of E6* short isoform (Williams et al., 2014). Genome-wide instability results in gradual inactivation of the repair system genes; the cell acquires a mutator phenotype noted for a sharp rise in the number of irreparable mutations affecting the functions of cell proto-oncogenes and/or tumor suppressor genes (Korzeniewski et al., 2011). Normally, the presence of such mutations would initiate the internal apoptotic pathway, but in the presence of E7 the replication process would continue even with such genome aberrations as double-strand breaks, translocations, inversions, insertions or deletions of extensive regions of chromosomes. The assumption that HPV-dependent oncotransformation is preceded by a prolonged stage of accumulation of mutations is indirectly corroborated by the usually long time period from the moment of infection to emergence of the tumor (Ibeanu, 2011).

Evidence of a high genomic instability, early activation of genome destabilization processes and their intensification with CIN and CeCa progression have been yielded by fluorescence techniques (DBD-FISH, DNA comet assay, etc.). The number of DNA single- and double-strand breaks in HPV-infected cells may rise already at the low-grade intraepithelial lesions (CIN1) (Cortes-Gutierrez et al., 2012). Presumably, it is the higher frequency of DNA breaks that increases the probability of

the viral genome integration into the epithelial cell genome (de Los Santos-Munive and Alonso-Avelino, 2013), which is the main stimulus for the CIN1 to CIN2/3 transition. Thus, HPV-DNA integration can be regarded as the insertional mutation that triggers most CeCa cases. Indeed, the portion of the integrated HPV-DNA form grows significantly with aggravation of the intraepithelial lesion, and can reach 100% in cases of cancer. Nonetheless, in a great number of cases the virus in tumor cells remains in the episomal state, suggesting cervical carcinogenesis can be driven by various mechanisms-both in association with the integration process and without such connection (Xu et al., 2013). The contribution of HPV physical status to the formation of the CeCa molecular-genetic '*portrait*' and the tumor cells phenotype is still undetermined.

CeCa development involves large-scope genome rearrangements affecting multiple genes. Several research teams identified changes in the number of copies of certain chromosome regions typical of CeCa development and progression. A substantial part of such aberrations is amplifications of genome regions ('*gains*'). The loss of genome segments ('*losses*') is far rarer in the case of CeCa compared to other types of cancers (Lee et al., 2012). Researchers are looking for CeCa stage-specific chromosomal aberrations; for instance, Oh et al. (2012) spotted the chromosomal abnormalities that differentiate CIN2 from CIN1 (5q35.3), CIN3 from CIN2 (2q14.3), and for invasive CeCa the authors described a much wider spectrum of unique rearrangements in chromosomes 1, 3, 5, 7 and 13. Wilting et al. (2009) reported on a CIN2/3-specific '*genomic signature*': the most frequent alterations were 'gains' on chromosomes 1, 3, 7, 20 and 'losses' on chromosomes 4, 11, 16, 17, 19, with the profile of genomic abnormalities in some CIN2/3 samples ('*gains*' on chromosomes 1, 3q, 20) similar to that in CeCa samples. From these data the researchers concluded that the 'signature' may point to a high probability of CIN2/3 progression to invasive cancer within a short time period. Oh and co-workers (2012) also discovered chromosomal alterations that persisted in the course of CIN and CeCa progression. The researchers believe the presence of differential and conservative aberrations confirms the hypothesis that CeCa progresses through repetitive rounds of clonal selection.

Meta-analysis of published data carried out by Thomas et al. (2014) showed that the most frequent chromosomal aberration in HPV16-positive CeCa and CIN2/3 was a 'gain' at 3q25-3q29. This region contains genes of relevance for tumor development, such as telomerase RNA component (TERC) and the PI3-kinase catalytic subunit gene. Supposedly, their amplification may perform the triggering function in the progression of high-grade intraepithelial neoplasia to true cancer (Zhao et al., 2015). Data obtained by Lee and colleagues (2012) corroborate this assumption. In this region the researchers found the gene of IVNS1ABP protein, which stabilizes actin microfilaments in the process of cell division and possesses anti-apoptotic properties. On the whole, as follows from the work of Senchenko et al. (2013) conducted with the use of NotI-microarray technology, on the course of CeCa development chromosome 3 undergoes profound structural changes (both genetic and epigenetic) affecting about 30 genes, significant part of which function as tumor suppressors. Among functionally significant deletions one should also single out aberrations on chromosome 6 in the region containing MHC I genes (6p21.3). The loss of heterozygosity (LOH) of the 6p21.3 region is an early event, as it is observed at the CIN stage and is coupled with a downregulation/loss of MHC-I gene expression. LOH frequency rises notably along the CIN-microcarcinoma-IB stage sequence, as well as in CIN regions surrounding the tumor locus, therefore researchers believe this genetic disorder facilitates immune evasion and is an indicator of unfavourable prognosis in CIN (Vermeulen et al., 2005; Mazurenko et al., 2006).

Another fact pointing to a high rate of genome destabilization processes in CeCa is that they involve also mitochondrial DNA (mtDNA): the number of mtDNA copies in CIN and CeCa cells increases, point nucleotide replacements and deletions of mtDNA segments become more frequent, which may be related to increased generation of active oxygen species (Warowicka et al., 2013).

Although the role of somatic point mutations inducing hyperactivation of cellular proto-oncogenes in CeCa development appears to be minor, such mutations can be found in some cases of progressing CeCa. Samples of stage I-IV CeCa in a study by Wright and co-authors (2013) were found to have activating mutations in PIK3CA (31.3% of cases), KRAS (8.8%) and EGFR (3.8%). Other point substitutions were much rarer. Furthermore, KRAS mutations were characteristic of adenocarcinoma, and EGFR mutations-of squamous cell carcinoma. The role of these mutations at the CIN malignization stage is still unknown. Given the comparatively low occurrence rate they probably should not be regarded as triggers, but identification of these mutations can help properly select targeted therapy (e.g., application of mTOR inhibitors in the case of hyperactivation of the PIK3K/Akt-signaling pathway).

Generally speaking, an extensive amount of data has lately been amassed on the problem of genome aberrations related to CIN progression and CeCa development. Application of the high-throughput sequencing technology (Akagi et al., 2014; Ojesina et al., 2014) helped reveal a much wider spectrum of mutations in CeCa cells than hybridization techniques (multiple inversions, translocations, duplications). However, these data need to be further verified, as they exhibit high variation, which can be partially explained by differences in methodology (e.g., analysis using FFPE tissue blocks, where genomic DNA may be partly degraded) (Lee et al., 2012). On the other hand, HPV-induced genome destabilization processes are non-directional and random, making it rather difficult to find the specific pattern of genome aberrations. Furthermore, the biological significance and clinical consequences of most CIN/CeCa-associated chromosome aberrations remain largely unknown (Narayan and Murty, 2010). Regarding early CeCa stages (IB-II) there is some experimental proof that the profile of changes at the genome level, namely an increase of a 'gene dosage', can partly predetermine the change of the genetic expression

profile (Narayan et al., 2007; Lando et al., 2009; Medina-Martinez et al., 2014).

Gene Expression Profile (Transcriptome)

More and more studies have lately been employing high-throughput (next-generation) sequencing (Peng et al., 2015) and cDNA microarrays (Luo et al., 2015; Rotondo et al., 2015) to compare gene expression profiles in normal cervical epithelial cells and in CIN/CeCa cells. CIN/CeCa development was found to be accompanied by regular changes in the transcriptome (Chao et al., 2007) caused primarily by specific features of HPV genome functioning. This fact has been proven by numerous studies on the profiling of model cell lines differing in HPV genotype, its physical status, integration sites, number of copies, or HPV oncogene expression, as well as of HPV-transfected keratinocytes or HPV-negative tumor lines (Min et al., 2009; Kaczkowski et al., 2012). Some researchers believe however that growing genome instability is a weightier factor for the gross transcriptome changes detected in CIN3 and CeCa than viral oncogene expression (Sopov et al., 2004; Lando et al., 2009).

Relying on the results described in the literature one can assume there exists an expression signature specific to normal epithelium, CIN1, CIN2, CIN3 and CeCa, and it can be used to build a molecular classification of CeCa and differentiate between its developmental stages, beginning from early intraepithelial changes. However, although the above techniques are highly informative and sensitive, the data obtained by different research teams show rather low reproducibility. It can probably be explained by the small sample size (few patients) due to high costs and high molecular heterogeneity of the pathology (Sgarlato et al., 2005).

Using the laser capture microdissection technique in combination with cDNA microarrays Gius et al. (2007) managed to thoroughly study mRNA profiles in normal and HPV-infected basal cells, CIN 1, 2 & 3 cells, invasive cancer and underlying connective tissue cells. Relying on their data the researchers suggested a model of neoplasia development through a succession of genetic and phenotypic signatures with regard to the close epithelium/stroma interaction: A) a '*pro-proliferative/immunosuppressive*' gene signature matches CIN1; B) a 'pro-angiogenic' signature matches CIN2; C) a 'pro-invasive' signature matches CIN3 and microinvasive CeCa. The pro-angiogenic and pro-invasive phenotype is formed through bilateral interactions of stromal fibroblasts and cervical epithelium cells. Group A is made up of the genes encoding the proteins regulating the cell cycle (p16INK4a, CENPF, KIF23, ITGAV) and the proteins regulating the immune response to intracellular infection (IFNAR1, IL1RN). Presumably, group A genes help maintain the proliferative status of suprabasal cells and resist antiviral immune response at early stages of the infection. According to the authors, the expression of group B genes (HINT1, MAP2K7, DAB2, TBX19, KAL-1) changes in response to local hypoxia and a deficit of cell growth substrates. The authors labeled the response to cell 'overcrowding' as the cause of the transition to the pro-invasive phenotype. Group C was formed of the genes regulating cell morphology, mobility and interactions with the intercellular matrix, such as desmoglein, metalloproteinases, TWEAK receptor.

Stroma involvement in CIN3 progression has been corroborated also by Chen et al. (2003). The authors analysed RNA profiles for high-grade squamous intraepithelial lesions (HSILs) and CeCa as compared with normal epithelium or low-grade lesions (LSILs), and found that the transcripts (EST) that were upregulated with the disease progression in epithelial and stromal cells were distributed between two functional gene clusters-1) replication controlling genes (including MCM 4/6, ARK2, topoisomerase IIA, proto-oncogene b-Myb), and 2) genes encoding intercellular matrix proteins, cell adhesion receptors (collagen, laminin, fibronectin, osteonectin, mesothelin, claudin) or intercellular matrix remodelling/degradation enzymes (matrix metalloproteinases, urokinase, etc.). Chen and co-authors communicated also that groups differing in expression patterns can be distinguished among these genes: some transcripts were upregulated gradually and more notably with LSIL progression to HSIL, whereas other genes were upregulated only in the case of CeCa. A study by Rajkumar et al. (2011) is also devoted to the analysis of how the transcriptome changed as the neoplasia progressed from CIN1 to CeCa. The authors distinguished a bigger number of gene groups differing in expression patterns: 1) genes with expression changing (decreasing/increasing) sharply in progression from normal epithelium to CIN1 and then retaining at that level across CIN grades and in progression to CeCa; 2) genes with expression changing sharply in CIN3 and retained at the same level in CeCa; 3) genes changing their activity only in CeCa cells; 4) genes that are the most active in CIN, whereas their expression in CeCa is suppressed; 5) genes with a complex bi-phasic change of expression, e.g., a rise in CIN1/2, a drop in CIN3/CIS (carcinoma *in situ*) and a significant rise in invasive CeCa. A fluctuating pattern of expression (increasing at the pre-invasive stage and decreasing at the invasive carcinoma stage) in some genes was observed also by Mattarocci et al. (2014). Rajkumar and co-workers (2011) employed bioinformatics tools to assemble genes with differential expression into a common regulatory network linked together by cellular transcription factors and viral oncogenes. The results of their analysis brought the authors to a conclusion concerning the cellular signaling cascades involved in malignant transformation and CeCa development: 1) interferon-induced signaling; 2) ubiquitin-dependent pathway; 3) cell cycle regulation pathways; 4) Myc-dependent signaling pathway; 5) E6/E7-dependent processes; 6) signals related to RNA metabolism; 7) p53-dependent mechanisms. Similarly to Rajkumar et al. (2011), other researchers also emphasized that when analyzing CeCa-associated changes in the transcriptome one should differentiate between disrupted expression of transcription factors (such as KLF4, Ahr-Arnt, c-Fos, c-MYB, E2F, Elk-1, Nrf, SPI-B, IRF, RUNX1, YY1 and ZNF143) and disrupted expression of their numerous target genes (Srivastava et al., 2014). Of particular interest are the transcription factors that are

typical of the stem cell transcriptome or drive stem cell renewal or differentiation, since they are the ones that may be responsible for the high phenotypic plasticity of tumor cells and the proliferative capacity (Organista-Nava et al., 2014), e.g. HOXB (Gonzalez-Herrera et al., 2015) and FOXC2 (Zheng et al., 2014) genes.

All the above data corroborate the current concept that carcinogenesis is a highly regulated multistep process, and the CIN1/CeCa stage 0-I transition, as an *in vivo* model, is quite illustrative for it. Transcriptome study in a progressing CeCa (FIGO stages IB-IV) is faced with some difficulties, the main one being high tumor heterogeneity (together with a significant proportion of immune infiltrate and necrotizing tissue, dense vasculature and stroma). Given these hindrances, Thomas et al. (2013) analyzed the change in the gene expression profile in the transition from CeCa 'early' stages (FIGO stages I-IIA) to 'late' stages (FIGO stages IIB-IV). According to their results, deregulation of over 80% of genes happened at CeCa early stages and was maintained with progression of the disease. One can thus assume that the phenotypic characteristics of the tumor are formed very early, due to the viral nature of the carcinogenesis. That is why Thomas and co-authors identified a minor number of genes specific to 'early' and 'late' FIGO stages. A more detailed further analysis showed that while individual genes deregulated in the 'early' and the 'late' stages were different, these genes still shared similar functional categories, i.e. the differential signatures of early and late FIGO CeCa stages were not specific to the functional load (Thomas et al., 2013).

CeCa stands out among tumors for its high metastatic activity, rapid formation of chemo/radio resistance and, hence, a poor prognosis. Naturally, researchers are eager to know the groups of genes whose activity is responsible for CeCa aggressive behavior. Lyng et al. (2006) carried out a comparative analysis of gene expression profiles in primary tumors from patients with positive and negative regional lymph nodes, and identified 31 genes whose activity correlated with lymph node involvement in the metastatic process. The genes were clustered into two groups depending on the co-linearity of change in their expression: the first group was associated with cellular metabolism adaptation to hypoxia (PDK2, KLF3), and the second one (TBX3, CKS2, etc.) portrayed a 'proliferative' phenotype and was closely associated with the tumor size. Rosty and colleagues (2005) also detected a 'proliferation cluster' associated with an early relapse and composed of 163 transcripts. Around 50% of them were targets of E2F transcription factor, which is directly activated by oncogene E7. Wong et al. (2003) identified a group of genes associated with patients' resistance to radiotherapy and encoding the DNA-repair system proteins, transcription factors, cytoskeletal components, and Ras family proteins.

Recent studies (Xu et al., 2013; Akagi et al., 2014; Shin et al., 2014; Hu et al., 2015) have demonstrated that when designing a molecular classification of CeCa based on genetic expression profiles one should take into account the different patterns of HPV integration into the host genome. Although it shows some preference for common fragile sites and transcriptionally active chromatin regions, HPV can be integrated into random sites of practically any host chromosomes (Xu et al., 2013). Next-generation sequencing of virus-enriched host DNA sequences has demonstrated that at least half of the samples had integration sites within or around structural genes, including key proto-oncogenes (MYC, ERBB2, FHIT, MECOM, BCAR4, POU5F1B, KLF5,12, HMGA2, LRP1B, SEMA3D) (Xu et al., 2013; Hu et al., 2015). Akagi et al. (2014) then found that HPV integrants immediately flanked the sites of genomic structural rearrangements, and could deregulate gene expression. Hu and co-authors (2015) also reported on down-regulated expression from FHIT and LRP1B genes when HPV integrated in their introns. The assumption that CeCa cell transcriptome and some clinical parameters depend on the pattern of HPV integration *in vivo* is corroborated by the results obtained by Shin et al. (2014). They distinguished 4 types of tumors with: 1) a single HPV-integrant copy; 2) a single HPV-integrant copy + episomal copies; 3) multiple integrated HPV-DNA copies arranged as tandem repeats; 4) tumors with negligibly low HPV-DNA number. Over 600 genes were differentially expressed in these groups, and the researchers attributed this fact to distinctions in the relative amount of E2/E6 proteins, which depend on the type of integration.

Another study carried out by Sveen et al. (2014) deserves attention as it describes the phenomenon of transcriptome instability, i.e. genome-wide variation in amounts of aberrant inclusion and skipping of exons due to disrupted pre-mRNA splicing process, in the development of CeCa. The range of CeCa transcriptome instability was revealed to be strongly (and inversely) associated with the expression of splicing factors and thought to explain, at least partially, the high level of molecular heterogeneity of CeCa and the existing discrepancies in transcriptomic research data. CeCa-specific altered expression of alternative splicing variants of a set of genes was also established by Guo et al. (2015) using RNA sequencing.

Profile of Epigenetic Modifications

Researchers' attention has lately been increasingly focused on the problem of epigenetic regulation of gene expression during CeCa development. HPV was found to induce large-scale changes in the 'landscape' of epigenetic modifications throughout the infected cell genome by hyperactivating or, on the contrary, silencing distinct sets of genes (Duenas-Gonzalez et al., 2005). The epigenetic level may well be the leading one in regulating the virus life cycle and neoplasia progression, but there is still no common opinion on the stability of epigenetic changes and reproducibility of *in vivo* data, on the specificity/sensitivity of potential epigenetic markers and the rate of their involvement in carcinogenesis and CeCa progression, as well as on prognostic power of the proposed epigenetic signatures (How et al., 2015).

Methylation profile (methylome): The best studied variants of epigenetic modifications in CIN/CeCa are: a) methylation of CpG sequences of gene regulatory regions; b) covalent modification of histones (Fang et al., 2014). Viral oncogenes can-through p53-/E2F-mediated

mechanisms or direct protein-to-protein interactions-induce overexpression or activation of cellular DNA methyltransferases (DNMTs), with the DNA methylation landscape substantially modified as a result (Jimenez-Wences et al., 2014). Promoters of tumor suppressor genes and repair genes in CeCa cells were found to be hypermethylated, wherefore their expression was inactivated (Saavedra et al., 2012; Fang et al., 2014); e.g., hypermethylation of FHIT suppressor gene (which contains one of the preferential sites for HPV16 integration) was confirmed to be related to HPV16 infection and CIN/CeCa progression (Bai et al., 2014). Simultaneously, as neoplasia progressed, the level of demethylation of cellular proto-oncogenes and anti-apoptotic genes increased, resulting in their overexpression. The scale of methylome change during CIN/CeCa progression is illustrated by the results obtained by Vidal et al. (2014), showing that the methylation pattern changed even for imprinted '*embryonic*' genes (IGF2, H19, PEG1/MEST), which normally feature the most stable CpG status. It was also discovered that epigenetic modifications could spread to various repeat elements of genome: so, for example, 3.3 kb-repeats located in subtelomeric regions of chromosomes 4 and 10 in more than 50% of CeCa cases were found to be hypermethylated, while satellite Sat2-sequences were hypomethylated, as compared with normal epithelium (Katargin et al., 2009), but how these changes contribute to CeCa development is not yet elucidated.

Although the methylome is highly dynamic and difficult to analyze, researchers have been trying to establish the CeCa-specific pattern and analyze its functional significance (effect on the expression levels of certain genes or activity of signaling cascades). Two independent studies by Chen et al. (2014) and Hansel et al. (2014) describe a CIN3+-specific signature marking the onset of an irreversible malignant process. According to Chen and co-authors (2014), it contains genes controlling extracellular matrix remodeling, cell adhesion, intracellular traffic, receptors of mitogenic stimuli, transcription factors regulating epithelial differentiation. The aberrant methylation of these genes in CIN3+ lesions compared to normal tissue is associated with a modification of their expression profile and tumor development. The most CIN3+-sensitive and specific gene was the relatively poorly studied POU4F3, encoding the homeobox family transcription factor. Among more than 100 candidate loci, Hansel et al. (2014) verified 5 marker genes associated with CeCa progression (CIN3+)-transcription factors DLX1, SOX17 and ZNF671, integrin ITGA4 and RXFP3 insulin-like receptor. The most CIN3+-specific among them was ZNF671. The results of both studies point to an essential role of epigenetic regulation mechanisms in disrupting the Wnt/β-catenin signaling axis during CeCa development. We know that the Wnt pathway controls cellular morphology, polarity, migration of cells, and is involved in the epithelial-mesenchymal transition (EMT) of transformed cells (Chen et al., 2014; Hansel et al., 2014).

CIN/CeCa progression is noted for a misbalance between the activities of two major histone modification enzymes-histone acetyltransferase HAT and histone deacetylase HDAC. HDAC upregulation in CIN and CeCa cells additionally contributes to the inhibition of activity (epigenetic silencing) of apoptotic genes, tumor suppressor genes, differentiation factors, namely p21Cip1/WAF1, components of the DNA-repair system (MGMT), retinoic acid receptor, E-cadherin (Saavedra et al., 2012; Feng et al., 2013). The promoters of some genes may simultaneously undergo methylation and deacetylation of associated histones, leading to sustained repression of these genes, but in CeCa such interaction of these two epigenetic mechanisms has so far been observed for only a few genes (e.g., retinoic acid receptor RARβ2) (Feng et al., 2013; Fang et al., 2014). The capacity of HDAC inhibitors to control tumor growth and HDAC potential as a therapeutic target are being studied in CeCa model systems.

HPV genome also undergoes epigenetic modifications (Johannsen and Lambert, 2013). The HPV-DNA methylation pattern depends on the life cycle of the virus, the extrachromosomal/integrated status, the activity of methyltransferases and the level of host cell differentiation, the disease stage. Modification of the methylation pattern by the intracellular microenvironment can result in HPV genome repression and rapid clearance on the one hand, or in aberrant expression of HPV oncogenes and malignant transformation on the other (Doorbar et al., 2012). Numerous studies overviewed by Johannsen and Lambert (2013) generally confirmed that the hypermethylation of HPV genome regions E2, L1, L2, which maintain the productive life cycle of the virus, increased during CIN to CeCa transition. In view of these patterns, HPV methylation profile can be regarded as a potential indicator of neoplastic progression (Brandsma et al., 2014).

Non-coding RNA (miRNA, lncRNA) profile: Small non-coding RNA (miRNA, miR) play a central role in regulating gene expression in eukaryotic cells. They can possess proto-oncogenic properties (onco-miRNA) or tumor suppressor properties, depending on the target gene functions. Suppressor miRNA are usually down-regulated during carcinogenesis, whereas proto-oncogenic miRNA are, on the contrary, upregulated. E.g., CeCa development is accompanied by a down-regulation of suppressor miR-218, the target of which is mRNA of laminin-5β3-an invasion marker (Saavedra et al., 2012); hyperexpression of proto-oncogenic miR-182 leads to degradation of mRNA of the FOXO1 factor, which activates p21 and p27 transcription, and thus suppresses apoptosis and stimulates the cell cycle (Tang et al., 2013). In another study, the expression level of miR-20a targeting TIMP2 metalloproteinase inhibitor was found to be significantly higher in cervical cancer patients than in healthy controls, while that of miR-203 (a miRNA that is important for keratinocyte differentiation) was lower (Zhao et al., 2013). The essential role of miRNA in shaping the CeCa cell phenotype is evidenced also by the results gained by Zhou et al. (2013), who found that knockdown of a key miRNA processing enzyme (Drosha) and the resultant proteome modifications inhibit cell proliferation and migration capacity.

During cervical carcinogenesis the expression of both

miRNAs associated with tumors of a different histological origin (e.g., lung, colorectal, breast cancer) and miRNAs specific to HPV-induced carcinogenesis may change. HPV oncogenes can employ various mechanisms to modulate the activity of miRNA genes (Zheng and Wang, 2011). Many of these deregulating effects are due to the E6-dependent degradation of the p53 protein, which is the transcription factor for many miRNA genes (Gomez-Gomez et al., 2013). Thus, the absence of p53 causes a down-regulation of miR-23b, which regulates the mRNA level of uPA plasminogen activator-a key inducer of invasion and metastasis. Another transcriptional target of p53 is miR-34a, which controls the expression of pivotal cell cycle and apoptosis regulators (Cyclin E2, Cyclin D1, CDK4, CDK6, E2F1, E2F3, E2F5, SIRT1, p18Ink4c, Bcl-2). The effect of E7 oncogene on miRNA is associated with the activation of E2F family transcription factors through E7-dependent pRb degradation. miRNA expression can also be deregulated by HPV genome integration into an immediate vicinity of their genes (Gomez-Gomez et al., 2013) or by chromosomal aberrations ('*gains*'/'*losses*') (Wilting et al., 2013). Finally, miRNA genes, similarly to protein-coding structural genes, are subject to epigenetic regulation. Thus, transcriptional repression of tumor suppressor miRNAs observed in CeCa can be a result of aberrant hypermethylation (Wilting et al., 2013a; Banno et al., 2014; Jimenez-Wences et al., 2014). A number of authors have published CIN/CeCa-associated miRNA signatures, but their structures varied widely. This variation was attributed to high miRNA metabolic rate, dependence of the miRNA profile on the HPV genotype (Gomez-Gomez et al., 2013), and the histological subtype of the tumor (Gocze et al., 2013; Banno et al., 2014). What makes changes in the miRNA profile difficult to interpret is that a single miRNA usually has dozens/hundreds of mRNA targets.

The microarray technique enabled identification of different miRNA clusters that gradually increased/decreased in amount as cervical neoplasia progressed (Saavedra et al., 2012; Wilting et al., 2013), but especially noteworthy are miRNA with a non-linear (stage-specific) expression pattern. For instance, Saavedra et al. (2012) detected miRNA that increased sharply in CIN3 lesions but recovered their normal expression during further transition to invasive CeCa. According to Wilting et al. (2013), among 106 differentially expressed miRNA, 27 were associated with malignant transformation and specifically marked CIN2/3 (their expression returning to normal afterwards), and 46 were specific to CeCa and not found at pre-invasive stages. Apparently, such a complex pattern of miRNA content temporal regulation indicates their involvement in 'fine tuning' of gene expression related to alteration of the genetic and phenotypic profile ('*portrait*') of CIN/CeCa as the pathology progresses. The results of comprehensive analysis of miRNAs-mRNAs expression profiles performed by Mo and co-authors (2015) for normal, CIN I, and CIN III epithelium samples provide convincing data in support of this statement. The authors applied SIG++ algorithm to detect the specific miRNA-mRNA pairs with significant regulation change and to construct miRNA differential regulatory network for different steps of CIN progression. The pathway enrichment analysis of '*efficient*' miRNA-mRNA pairs demonstrated that, for CIN1, the specific miRNA-mRNA regulations were highly enriched in cell migration and keratinocyte differentiation; for CIN3, the specific miRNA-mRNA regulations were enriched in virus integration; and for each stage these regulations were enriched in inflammation and angiogenesis (Mo et al., 2015).

Researchers have also tried to determine the spectrum of miRNA that control the hallmark of CeCa aggressive behavior-the capacity for rapid invasion and early metastasis. Ding et al. (2014) compared the pools of tumor miRNA in lymph node positive and negative patients, and identified 39 differentially expressed miRNA, to which they applied qPCR and verified miR-126, miR-96, miR-144, miR-657, miR-490-5p, miR-323-3p. The authors analyzed potential mRNA targets of the verified miRNA: they turned out to include genes responsible for intercellular adhesion, migration and cytoskeletal rearrangement (namely matrix metalloproteinases, fibronectins, cortical cytoplasm linker proteins, receptors of extracellular matrix proteins). Yu et al. (2014) established the role for miR-126 in drug resistance of CeCa. Wang et al. (2013) also observed significant up-regulation of miR-93 and miR-200a that was associated with metastasis and invasion of cervical carcinoma. These results suggest that miRNA take part in building the metastatic potential of CeCa. It has also been reported in recent publications that the profile of serum (secreted) miRNA may change early in cervical carcinogenesis to largely overlap the tissue miRNA profile, indicating the presence of metastatic lesions in lymph nodes (Chen et al., 2013; Gocze et al., 2013; Banno et al., 2014). Bioinformatic analysis confirms the involvement of CeCa-specific serum miRNA in regulating cell migration, invasion and metastasis.

Quite interesting is the recent discovery of HPV-coded miRNA, enabled by deep sequencing of small RNA libraries (Qian et al., 2013). The spectrum of potential cellular targets of the identified viral miRNA was made up of the genes that regulate the cell cycle, especially the M phase, epithelial differentiation, T-cell immune response, intercellular adhesion, migration, activity of redox processes. Thus, the profile of HPV-specific miRNAs is functionally intertwined with the profile of cellular miRNA, making it far more difficult to understand the mechanisms employed by the virus to fulfill its genetic programme.

Modification of the expression profile of long non-coding RNA (lncRNA) in tumor cells is also of high interest for researchers. Gibb et al. (2012) employed deep sequencing to obtain CIN1/2/3-associated expression profiles of 1056 lncRNA-transcripts. The authors observed that intraepithelial lesions (especially CIN3) featured an aberrant expression of these unique mRNA-like molecules, but the biological role of lncRNAs and their contribution to the transformation and progression of the neoplasia remain unknown.

Protein Profile (Proteome)

The final outcome of the genetic and epigenetic lesions induced by HPV oncogenes is an alteration of cellular protein profile, i.e. the proteome. To verify the viral background of cellular proteome aberrations, Higareda-Almaraz et al. (2011) carried out a comparative analysis of the proteomes of six CeCa cell lines and the non-tumorigenic human keratinocyte line HaCaT using 2D gel electrophoresis and MALDI-TOF mass spectrometry. The authors reported of 66 proteins (called the '*central core of CeCa*') the expression of which changed sharply in all CeCa cell lines as compared with normal keratinocytes, and classified them into the following functional groups: 1) proteins involved in cell migration, adhesion, epithelial-mesenchymal transition and metastasis (namely, vimentin, vinculin, ezrin, galectin-1, annexin 2, protein disulfide-isomerase); 2) proteins related to evasion of apoptosis (including members of the chaperone Hsp70 family); 3) energy metabolism enzymes (including glycolytic enzymes, LDH). Their findings suggest also that 14-3-3ζ proteins play a central part in determining the '*fate*' of an infected epithelial cell. 14-3-3 proteins are universal adaptors of protein-protein interactions, and appear to be a crucial hub for the proteins pathologically expressed in CeCa cells (Higareda-Almaraz et al., 2011).

Papers (Bae et al., 2005; Lomnytska et al., 2010) describe the results of investigating the profiles of proteins extracted from biopsies. Both research teams reported a profound change in the CeCa cell proteome compared to healthy tissue, namely a deregulation of cytoskeleton proteins (cytokeratins, tropomyosin), chaperons, surface proteoglycans, annexins, apolipoproteins, etc. An essential component of the proteome of cervical epithelial cells is proteins responsible for intercellular adhesion ('*adherens junctions*' and *'tight junctions'*) and apical-basolateral polarity. Impaired functioning of these proteins is thought to be associated with the epithelial-mesenchymal transition (EMT), which is now regarded as a turning point in CeCa progression. Indeed, Cunniffe et al. (2012) showed that the development of CIN lesions and an early invasive process is associated with a loss of E-cadherin, claudins, occludins, as well as aberrant expression of the transcription factors that specifically regulate intercellular adhesion and cell interactions with the extracellular matrix (e.g., Snail). Information about the role of certain proteins in invasion and metastasis can be obtained by comparing the proteomes of tumor samples from patients with or without metastatic loci in regional lymph nodes. Wang et al. (2014) reported of three proteins whose content correlated with the presence of micrometastases at early stages of cervical carcinogenesis-FABP5, HspB1 and MnSOD; these proteins have been shown to take part in regulation of keratinocyte proliferation and differentiation, cytoskeleton rearrangement, NFkB pathway activation, control over the level of free radial oxidation.

A special issue in the analysis of CeCa-associated proteome alterations is changes in the cell metabolome and degradome (Rolen et al., 2006; Lou and Wang, 2014). We are learning that HPV actively uses ubiquitination systems in cells (various ubiquitin ligases and deubiquitinases) and influences their functioning through E6/E7 oncogenes. Modification of the activity of ubiquitination systems or availability of their targets under the effect of HPV proteins may lead to a slower circulation of proto-oncogenic factors and their accumulation in the cell or, vice versa, to accelerated degradation of tumor suppressors. Alteration of the cellular proteome leads also to changes in the glycome-the profile of oligosaccharide antigens in glycoproteins and glycolipids. The expression of various glycosyl transferases in CeCa cell is deregulated, resulting in aberrant sialylation and fucosylation of cytoplasmic proteins (Kim et al., 2014; Rivera-Juarez et al., 2014), which are not glycosylated in normal cells. Apparently, such modifications affect the functional activity of these proteins. Hyperexpression of O-glycosylated proteins on the surface of CeCa cells promotes their invasive capacity by rearranging intercellular interactions (Solorzano et al., 2012). Sialylation of surface antigens, namely Lewis antigens, which are responsible for tumor cell adhesion to the endothelium, is promoted (Velazquez-Marquez et al., 2012). Formation of the immune suppression microenvironment by modifying the spectrum of secreted cytokines and their receptors, which enables the virus and HPV-transformed cells to evade immune surveillance, can also be considered as a component part of cellular proteome changes (comprehensively reviewed by Paradkar et al. (2014)).

Conclusions

The concept of step-wise cervical carcinogenesis, initially based on pathomorphological observations, is continuously gaining corroborating evidence from studies that employ genome-wide screening of molecular disorders at different stages of the disease. Each molecular profile (genome, transcriptome, proteome, etc.) evolves, gradually accruing multiple aberrations and thus facilitating the construction of an integrated model of tumor development. The mechanisms for CeCa progression can be both general, characteristic of carcinogenesis as such, and CeCa-specific. As high-throughput research techniques and methods for the analysis of large bodies of biological data are developing, the cervical carcinogenesis model is being constantly updated. For instance, recent findings have brought a new understanding of the role of antiviral immune response in the neoplasia progression: if immune response elicits at the productive infection phase, it will result in clearance of the virus and regression of the dysplasia locus, but if immune response is launched after HPV integration into the host cell genome, the immune system will, on the contrary, promote CIN progression, as it removes the episomal form of the virus and the negative control over oncogenes within HPV integrants (Mine et al., 2013; Shulzhenko et al., 2014). The same organism may display both inadequate immune response at the benign growth stage and its activation during keratinocyte transformation resulting from genomic rearrangements and other gene expression deregulation events (Shulzhenko et al., 2014).

In spite of the avalanche of new data on CeCa biology, many problems remain to be clarified. Modern molecular

profiling cannot reliably differentiate productive CIN1/2 from transforming CIN2/3, as well as distinguish between CIN3 lesions with a short and a long period of progression to invasive cancer (Steenbergen et al., 2014). The current cervical carcinogenesis model does not have sufficient predictive power, since the triggers of irreversible malignant transformation are not definitely known, and it is unclear why these triggering mechanisms work in a limited (and, in fact, minor) proportion of CIN lesions. Much difficulty arises from the high variability of the CeCa molecular-genetic '*portrait*', which appears to be its hallmark.

To comprehensively reconstruct the mechanism of cervical carcinogenesis and identify its driving forces, one should determine the links between the various layers of molecular lesions. Such an integrated approach is now widely applied to CeCa (papers by Narayan and Murty (2010); Higareda-Almaraz et al. (2013); Liang et al. (2014)). A technique that appears to be the most promising is next-generation sequencing (NGS) with its various applications that enable simultaneous analysis of the spectrum of somatic mutations and alterations in the exome, transcriptome, 'histone code' across the entire genome, i.e. integration of at least three regulatory levels (Liang et al., 2014). Analysis of deregulation patterns in functionally distinct groups of genes helps identify specific signaling pathways that stimulate tumorigenesis. Kaczkowski et al. (2012) argued that, given the highly controversial data on CeCa, research should be focused more on pro-oncogenic signaling cascades rather than individual genes or proteins. Halim et al. (2013) also remarked that since there are several signaling mechanisms behind each phenotypic characteristic of tumor cells, any impact directed at a specific molecular target is doomed to be ineffective. Thus, although fundamental in nature, studies devoted to drawing of the integrated molecular-genetic '*portrait*' of CeCa are meant to establish the background for targeted and efficient search for biomarkers for differential diagnosis and targets for multi-target therapy of this oncopathology.

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