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The effects of miRNA-21 on the epithelial to mesenchymal transition in cancer

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Abstract: MicroRNAs (miRNAs) are short, non-coding RNAs which regulate the post-transcriptional gene expression. They have been implicated in many cellular processes in physiological and pathological conditions, including cancer development and progression. miRNAs are important players in epithelial to mesenchymal transition (EMT), which is a phenomenon observed during tumour invasion and cancer metastasis. The most typical changes in gene expression profile occurring in EMT is down-regulation of epithelial genes, such as for E-cadherin, and up-regulation of mesenchymal genes, such as for N-cadherin and vimentin. miRNAs appear to be potentially useful in the clinical diagnostics and as a therapeutic targets. miRNA-21 regulates the cancer development in many ways, including control of genes expression associated with the EMT process.

1. Introduction

miRNAs regulate numerous, crucial biological processes. One of them is the epithelial to mesenchymal transition (EMT). Furthermore, miRNAs have been linked to many diseases, including cancer, and potentially can be useful for the clinical diagnosis [1]. For instance, in gastric cancer miR--18a, miR-106b, miR-21, miR-203, miR-146b, miR-192, and miR-200c are released to the plasma, therefore they can be a potential biomarkers of this cancer in specific ethnic group [2]. It is believed that exploring the functional importance of miRNAs in cancer development and progression may open a new ways in tumor treatment. The relationship between miRNA and cancerogenesis was well established for cluster of miR-17-92 in lymphomas and leukaemias, for miR-34 in neuroblastoma [1] as well as for miR-21 in breast cancer [3]. miRNAs which are associated with the development of cancer have been divided into two groups. In first of them, in which miRNAs are overexpressed, they are called oncogenic miRNAs (oncomiRNAs). In the second group they are underexpressed and named suppressive miRNAs (suppressor miRNAs). When the expression of the oncomiRNAs and suppressor miRNAs is upregulated or reduced, respectively, cancer cells proliferation and metastasis are induced [4].

There are many studies on the role of miRNAs in the cancer cells development and tumor progression. Since the great majority of death caused by cancer is due to metastasis formation in the vital organs, also the role of miRNAs in this phenomenon is intensively studied. In many types of cancer the process of EMT has an important role in the regulation of the metastasis by facilitating tumour cell invasion and dissemination to the distant organs. Currently, many clinical trials which modulate EMT process are ongoing. The knowledge on factors which regulate this process may lead to the development of a new strategy for treatment of cancer [5].

The initiation of EMT depends both, on the activation of activators of this process, and on the inactivation of inhibiting proteins. miRNAs, as a regulators of gene expression, may interfere with mRNA for inhibitory proteins and thus, contribute to the activation of the cascade leading to EMT. Many signaling pathways leading to the EMT are known, some of them are regulated by miR-21.

This paper is based on the review of the literature included in the PubMed database, using "EMT", "epithelial to mesenchymal transition", "cancer", "miRNA", "miR-21", as a key words.

2. miRNAs biogenesis and regulation of the gene expression

miRNAs are a group of small single-stranded non-coding RNAs that regulate the post-transcriptional gene expression. The first miRNA was discovered over 30 years ago during the study of a nematode, nowadays thousands of miRNAs are reported in many organisms [1]. In human genome, around 2600 miRNA genes are annotated [6]. They consist of 1-5% of all predicted human genes [7]. There is not a simple correlation between miRNA and mRNA expression, which means that multiple miRNAs can target the same mRNA and one miRNA can target many mRNA [8]. miRNAs

repress expression of the genes mainly by binding to complementary sequences in the 3' untranslated region (3'UTR) of mRNA. miRNA-mRNA interaction results latter degradation or translation inhibition [5]. miRNAs can be categorized into different groups, which are called miRNA families. The membership to specific miRNA family is determined by mature miRNA (described also as miR) and by the structure of precursor miRNA (pre-miRNA) [9]. All miRNAs undergo a series of biogenesis steps that convert the primary miRNA (pri-miRNA) transcript into pre-miRNA and finally into active, 20-25 nucleotide (nt) mature miRNA.

About half of all currently identified miRNAs are intragenic and processed mostly from the introns and relatively few exons of the protein coding genes, while the remaining are intergenic, transcribed independently of a host gene and regulated by their own promoters[10]. The biogenesis of miRNA takes place in a multi-step process. The first step occurs in the nuclear, and the second in the cytoplasmic compartment of cells. The most of miRNA genes are transcribed by RNA polymerase II (or less frequently by polymerase III), as a long pri-miRNAs of approximately 80 nt., with a cap and a poly-A tail. These pri-miRNAs having the shape of hairpin consist of a double-stranded (ds) stem of about 30 base pairs, a terminal loop and two flanking single-stranded tails [11]. pri-miRNA is processed in the cell nucleus by a heterotrimeric complex, named Microprocessor into short, approximately 70-nt. stem-loop structure known as pre-miRNA. The Microprocessor consists of the RNAse III enzyme, Drosha, and two molecules of its essential cofactor DiGeorge syndrome critical region 8 (DGCR8) [9]. Drosha contains a double stranded RNA-binding domain and two RNAse III domains. Each of RNAse III domains cleaves the 3' or 5' strand of a pri-miRNA hairpin, and as results pre-miRNA is formed [8].

In the next step pre-miRNA is transported from nucleus to the cytoplasm in a Ran-GTPase dependent manner by an export receptor, exportin 5 (EXP5), where it is further processed with the endoribonuclease III, named Dicer [1, 8]. The main functional domains of Dicer are: ATPase RNA helicase domain, a PAZ domain, two catalytic RNAse III domains, DUF283 domain, and C-terminal a double stranded RNA-binding domains [12]. Binding of Dicer by RNAse domains to the end of the pre-miRNA cuts off the dsRNA stem close to the terminal loop and produces 20-25 nt. long mature duplex of miRNA. In humans, DICER function together with the trans-activation responsive RNA-binding protein (TRBP), which enhances the precision of DICER-mediated cleavage of pre-miRNAs. Upon cleavage by Dicer, the short ds miRNA product is transferred onto an Argonaute family (AGO1-4) proteins in a process termed RNA inducing silencing complex (RISC) loading [13]. AGO consists of N-terminal domain, a PAZ domain, middle domain and PIWI domain, having activity of the endoribonuclease. Next, one strand of the ds pre-miRNA is cleaved by AGO protein and a single-stranded, matured mi-RNA is formed. The strand loaded into AGO is considered the guide strand, the unloaded strand is termed the passenger strand [10].

The important miRNA region of the guide strand interacting with mRNA is termed "seed region". It consists of two to seven nucleotides on the 5' end of miRNA. The further fate of mRNA depends on the complementarity between miRNA and mRNA. If a full complementarity exists, the target mRNA can by degradated via the AGO2, which possesses an endonucleolytic activity. This type of mRNA processing is seldom in animal cells. Most of the target mRNAs do not have fully matched sequence with miRNA, and therefore, cannot be directly cleaved by the AGO2. In such situation, 9-11 nucleotides of miRNA, together with a members of the TNRC6C/GW182 family of proteins, interact with the 3' UTR region of the target mRNA. These proteins are involved in inhibition of the translation of mRNA. This interaction occurs in the processing bodies (P-bodies), which are the cytoplasmic foci of the transcripts storage and degradation [1, 8].

3. miRNAs nomenclature

miRNA is called using the prefix "miR" and a numeric suffix, e.g. miR-21, miR-34. The three preceding letters denote the species. For humans (*Homo sapiens*) those letters are "hsa" (e.g. hsa-mir-21), for a mouse (*Mus musculus*) they are "mmu" (e.g. mmu-mir-21).

Evolutionary related miRNAs possess a letter after the number in the suffix, by this, multiple members of the same family can be differentiate (e.g. hsa-mir-34a and hsa-mir-34b). Identical mature products produced by two diverse loci are labeled by additional number after the full name. For instance, hsa-mir-1-1 and hsa-mir-1-2 produce the same final microRNA product: hsa-miR-1. A tag -3' or -5' added to the name indicate from which double-stranded RNA the mature sequence comes from (e.g. [R*attus norvegicus*] rno-miR-21-5p from the 5' arm of the precursor and rno-miR-21-3p from the 3' arm of the precursor). For the first miRNAs discovered, "let" and "lin" prefixes are used, instead of "miR" (e.g. let-7, lin-4) [14, 15].

4. Epithelial to mesenchymal transition is regulated by miRNAs in carcinogenesis

Epithelial to mesenchymal transition is a process in which epithelial cells lose cell-cell adhesion and polarity and gain a motile mesenchymal phenotype. This phenomenon can be consider as a continuum, whereby cells exhibit epithelial, transitional and finally mesenchymal phenotypes [16]. The major changes in gene expression profile occurring in EMT are associated with decreased expression of epithelial genes such as E-cadherin, mucin-1, cytokeratins, occludin as well as desmoplakin, and increased expression of mesenchymal genes such as, N-cadherin, vimentin, smooth muscle alpha actin (αSMA), fibronectin, and vitronectin. The EMT has been classified into three categories: type I, associated with embryogenesis; type II, connected with wound healing, what means associated with tissue regeneration and organ fibrosis; and type III, implicated in carcinogenesis and tumor progression [17].

The EMT confers the metastatic properties upon cancer cells by increasing mobility, invasion and resistance to the apoptotic stimuli [18]. Moreover, EMT closely associate with acquisition of stemness and therapy resistance [19, 20]. Regulation of the gene expression through miRs may contribute to both, the initiation of EMT process and its inhibition [21].

The EMT can be induced by many factors, such as Transforming Growth Factor beta (TGFβ), Fibroblast Growth Factor (FGF), Platelet-derived Growth Factor (PDGF), Epidermal Growth Factor (EGF), as well as Wnt and Notch signaling pathways [22]. Many transcription factors, including SNAIL1 (Zinc finger protein SNAIL1), SLUG (SNAIL2), Twist1/2, (ZEB)1/2 (Zinc finger E-Box-binding homeobox), HIF-1α (Hypoxia-Inducible Factor-1-alpha), E12/E47, Dlx-2 (Distal-less homeobox 2), and several regulatory molecules contribute to EMT [23].

One of the transcription factors important for EMT which may be regulated by miRNAs is Snail1. SNAIL1 is able to bind the E-box on E-cadherin gene promoter and to reduce this gene expression. Moreover, SNAIL1 is co-expressed with WNT3a (Wnt family member 3) protein, which is a master regulator of SNAIL1. Wnt3a inhibits SNAIL1 phosphorylation and increases SNAIL1 protein levels. One of miRNAs which can regulate the function of SNAIL1 is cluster miRNA-34 a/b/c located on chromosome 1. These miRNAs are considered as a tumour suppressors and are overexpressed in an epithelial state in EMT. SNAIL1 induces also the zinc-finger transcription factor 281 (ZNF281), involved in initiation of the mesenchymal phenotype in EMT. The zinc-finger transcription factor 281 suppresses the miRNA-34 at transcriptional level. This transcription factor-miRNA interaction provides a feedback loop [24, 25].

Another member of the zinc-finger transcription factors, engaged in EMT, and regulated by miRNAs is SNAIL2 (also known as a SLUG). SNAIL2 is one of the major EMT inducer, but it is a less potential suppressor of a E-cadherin than SNAIL1. It was shown that SNAIL2 expression correlate with a distant metastasis [26]. miR-203, coded by gene located on chromosome 14 interacts with SNAIL2. This miR is considered to be an anti-proliferative agent. The promoter region of miRNA-203 possesses a three different putative SNAIL2 binding sites. This interaction causes the suppression of miRNA-203 function. Transforming growth factor beta, induces EMT by interaction with SNAIL2 and also represses miRNA-203 function. There is a specific feedback loop in which miRNA-203 induces SNAIL2 repression, leading to the overexpression of E-cadherin and to the maintenance of epithelial phenotype [27, 28].

ZEB1 is the zinc-finger transcription factor, which suppresses of E-cadherin expression during carcinogenesis, and interacts with SMAD complex (SMAD1/2/3). Association between ZEB1 and SMAD may cause different activity of this transcription factor; from repression to co-activation of the transcription. Moreover, ZEB1 is a mediator of TGF-β signaling pathway, which is the major inducer of EMT. ZEB1 can be regulated by a potent EMT inhibitors such as a members of miRNA-200 family (miRNA-200a/b/c, miRNA-141, miRNA429) [29]. TGF- β is also target for miRNA-200. The controls of cell plasticity, between the epithelial and the mesenchymal states depends on autocrine TGF-β/ZEB1/miR-200 signaling network. The ZEB1 factor binds to the E-boxes in promoter region of E-cadherin gene and causes its transcriptional repression. It has be demonstrated also that ZEB1 binds to the E-boxes in miR-200 gene promoter, and thereby suppresses its expression. Therefore, while miR-200 causes post-transcriptional repression of ZEB, the latter regulates transcriptional repression of miRNA-200. These feedback mechanisms lead to the increased expression of TGF-β1 and TGF-β2, which is correlated with low miR-200 and high ZEB expression [30-32].

TWIST1 belongs to the class of alpha basic helix-loop-helix transcription factors [33], which also may be regulated by miRNAs. TWIST1 expression down-regulates the epithelial genes (e.g., for E-cadherin and claudin-7) and up-regulates genes for the mesenchymal proteins. TWIST1 is one of the downstream targets of let-7 miRNAs family, which includes let-7e-; and let-7b miRNAs as well as miRNA-98. In many cancers, let-7 miRNA family members, which possess anti-metastatic function, are significantly reduced. The biogenesis of let-7 miRNAs is regulated by RNA-

-binding proteins, LIN28A and LIN28B, which block DICER cleavage activity, and therefore inhibit the formation of these miRNAs. It was also shown that in many human cancers LIN28A/B are over-expressed. This leads to reduction of let-7 miRNA, and in consequence to TWIST up-regulation [34].

5. miR-21 (oncomiR) controls EMT in various types of cancer

miR-21 is important in regulation of such oncogenic processes as high cells proliferation and invasion, metastatic potential, and low apoptosis, therefore it is an example of oncomir [35]. miR-21 gene is located in the intronic region which overlaps with the 3' UTR end of the transmembrane protein 49 (TMEM 49) gene in q23.2 on chromosome 17 [36]. TMEM49 protein mediates autophagy and regulates cancer-relevant processes, such as an inhibition of proliferation and metastasis [37]. The overexpression of miR-21 is noted in glioblastoma and in many other types of tumors, including B-cell lymphoma, hepatocellular carcinoma and cancers of head and neck, breast, ovary, cervix as well as lung. Numerous targets for miR-21 have been described, e.g., PTEN, PDCD4, BTG2, HIF1α, TIMP3, TM1 [35, 38], LIF, STAT3 [39], NR2F2 and Smad7 [40]. It was demonstrated that TGF-β increases miR-21 expression in cancer cells, and causes induction of cancer stem cells-like phenotype, and also increases hypoxia-inducible factor 1 alpha (HIF1α) levels. This factor is known to be induced not only by the hypoxia, but also by the growth factors and oncogenes. In addition, there is relationship between miRNA expression and increased HIF1α expression, since miR21 targets PTEN, what leads to enhancement of the HIF1α level [41]. The main target for the tumor supressor PTEN (phosphatase and tensin homolog) is phosphatidylinositol 3,4,5 trisphosphate (PIP3). PIP3 recruits Ser/Thr kinase AKT to the plasma membrane, what leads to the phosphorylation and activation of AKT by phosphoinositide-dependent kinase-1 (PDK1). The direct phosphorylation target of AKT is the family of forkhead transcription factors (FOXO) [42]. One of them, namely, FOXO3a upregulates HIF-1α. miR21 via suppressing PTEN increases the level of PIP3 and HIF-1α as a result of upregulation of AKT-dependent activation of FOXO3a [43].

It is known that HIF-1α directs the expression of many EMT regulators and induces the loss of E-cadherin by transcriptional activation of genes encoding repressors of E-cadherin expression, such as ZFHX1B (Smad interacting-protein 1), ZFHX1A (Zinc Finger Homeobox Protein 1A) and TCF3 (Transcription factor 3) [44].

HiF-1α, by binding via hypoxia response element (HRE) sites in the Zeb1 or Twist1 proximal promotor, can regulate the expression these transcription factors [45-47].

In the breast cancer cells, overexpression of miR-21 promoted EMT by regulation of PTEN and AKT pathway [48].

Moreover, miR-21 has a crucial role in colorectal cancer. It was shown, that in colorectal cancer, miR-21 is upregulated in preneoplastic and neoplastic parenchymal and stromal cells as well as in the serum. It was also demonstrated that the level of miR-21 can be a predictor of tumour relapse and poor survival. In colorectal cancer cell line overexpression of miR-21 contributes to the lose of epithelial marker, such as E-cadherin, and to the acquisition of mesenchymal marker, N-cadherin. miR-21 promotes EMT probably by the regulation of NR2F2/TGF-β/SMAD7 pathway [49] and ITGβ4/PDCD4 network [50]. NR2F2 (Nuclear receptor subfamily 2 group F member 2) is commonly upregulated in cancer cells and is considered to be a key transcription factor in the development of breast, lung, prostate and colorectal cancers. Smad7 protein is a negative regulator of TGF-beta pathway. NR2F2 activates miR-21 expression by binding to its gene promoter. In turn, miR21 inhibits SMAD7 and therefore TGF-beta signaling cascade is activated. In addition, NR2F2 inhibits Smad7 expression and promotes TGF-β-dependent EMT [40].

ITGβ4 (Integrin-β4) plays a role in the regulation of EMT and is expressed in epithelial cells. Expression of the gene for this integrin is another target for miR-21. Modulation of ITGβ4 protein levels, by miR-21, is executed via repression of mRNA translation acting on 3' UTR regions, and also through mRNA degradation. Inhibition of miR-21 results in increased ITGβ4 mRNA expression and the protein level. It has been postulated that high miR-21 level combined with low ITGβ4 and PDCD4 (Programmed Cell Death 4) expression is able to predict the presence of colorectal cancer metastasis [50].

PDCD4 is a tumour suppressor and its upregulation is closely linked to apoptosis. Decreased PDCD4 expression enhances malignant transformation, by intensifying the expression of apoptosis inhibitors, and causes also chemoresistance by induction of multidrug resistance protein, MDR1/P-gp [51]. In addition, downregulation of PDCD4 leads to the low expression of epithelial-specific proteins (α-catenin and γ-catenin), and high expression of mesenchymal-specific proteins (N-cadherin and fibronectin), in HT29 colon cell line. The presumed mechanisms of these phenom-

ena are through activation of β-catenin dependent transcription. A decrease in E-cadherin expression, which is the binding partner of β-catenin, results in an increase of free β-catenins in the cytoplasm. Free β-catenins are phosphorylated by glycogen synthase kinase 3β (GSK3β) in the adenomatous polyposis coli (APC)-axin-GSK3β-casein kinase I complex, and next degradated. Mutations of the APC or block of GSK3β activity, cause translocation of β-catenin into nucleus and initiation of the β-catenin-dependent transcription. A downstream target of β-catenin-dependent transcription is c-Myc, transcription factor commonly up-regulated in tumor cells [52].

It is assumed, that PDCD4 is directly engaged in EMT regulation by inhibition of Snail translation, which is a master transcription factor for this transition. Therefore, downregulation of PDCD4 by miR-21 can lead to Snail overexpression and EMT initiation [52].

miRNA-21 expression may be associated with IL-6 (interleukin 6) upregulation. This interleukin is involved in a variety of phenomena, including the inflammatory response, oncogenesis, regulation of cell growth, survival, differentiation and many other processes. IL6 binding to its receptor (IL6R) leads to the activation of receptor-associated Janus kinases (JAKs), and following phosphorylation and dimerization of STAT3 (Signal transducer and activator of transcription 3), its translocation to the nucleus, and miRNA-21 gene expression [53].

miR-21, as many others molecules, can be loaded into vesicles (exosomes), which after exocytosis are able to integrate into surrounding cells, where they release functional miRNAs. Some data show, that cancer and immune cells cultured *in vitro* interact and cross-talk via IL6 and miRNAs. The tumor-associated immune cells produce IL-6, which binds to IL6R on the cancer cells surface. As a result, a number of oncogenes and miRNAs, including miR-21 is expressed. After secretion into environment, miR-21 is taken up by the tumor-associated immune cells endosomes and binds to TLR8 (Toll like-receptor-8). This interaction induces the NF-kB pathway and further IL-6 secretion [53].

Considering the uncontested functions of miR21 in tumorogenesis, it may be important to inhibit this molecule. A number of studies indicate that miR-21 gene editing and silencing may inhibit the phenomenon of EMT and cancer progression. An example can be application in ovarian cancer cell lines the Lentiviral CRISPR/Cas9 vectors, which mediate mutation in sequences of the miR-21 precursor. This results in upregulation of E-cadherin expression and downregulation of Snail2 and vimentin, and in consequence the reduction of cell proliferation, migration and invasion [43].

Application of miR-21 inhibitor in NSCLC (non-small cell lung cancer) cell line suppresses the phosphorylation of Akt and promotes apoptosis through inhibition of PI3K/Akt/NF-κB signaling pathway. Furthermore, in NSCLC cell down-regulation of miR-21suppresses cell migration and invasion, as well as EMT signaling pathways [44].

It has been documented also, that transfection of the breast cancer stem cell-like cells with human Hsa-miR-21 antagomir reverses EMT phenotype and HIF-1α expression, both of which are consistent with the tumor cells invasion and migration [36].

miR-21 is implicated in the drug resistance to neoadjuvant treatment in trastuzumab and chemotherapy, in HER2-positive breast cancer patients. The DNA damage induced by this therapy upregulates the expression of miR-21, by activating NF-κB, what consequently sustains EMT in breast cancer. Probably, the mechanism of resistance to trastuzumab-chemotherapy in patients with HER2-positive tumors depends on miR-21-mediated silencing of PTEN and PDCD4 proteins. The use of anti-miR-21 inhibitor in human breast cancer cell line leads to increase of susceptibility to trastuzumab, and to the reduction of viability of these cells. It has been postulated, that the increase in miR-21 expression in neoadjuvant trastuzumab-chemotherapy can be a predictive biomarker of resistance to this treatment [3].

6. Summary

miRNAs can regulate numerous biological processes, including EMT phenomenon during cancer development. This process contributes to the regulation of cells motion and in consequence to the cancer cells metastasize and tumor progression. In this review we illustrate mainly the role of miR-21, and some other selected microRNAs in the control of EMT process in cancerogenesis. The regulation of miRNAs is complicated and its specificity depends on cancer type. Understanding the molecular mechanisms, which are controlled by miRNAs may contribute to the generation of new strategies in therapy, and therefore to the life extension of patients with cancer.

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