Snail1 is a zinc-finger transcription factor, which plays a role in colorectal cancer development by silencing E-cadherin expression and inducing epithelial-mesenchymal transition (EMT). During EMT tumour cells acquire a mesenchymal phenotype that is responsible for their invasive activities. Consequently, Snail1 expression in colorectal cancer is usually associated with progression and metastasis. Some studies revealed that about 77% of colon cancer samples display Snail1 immunoreactivity both in activated fibroblasts and in carcinoma cells that have undergone EMT. Therefore, expression of this factor in the stroma may indicate how many cells possess the abilities to escape from the primary tumour mass, invade the basal lamina and colonise distant target organs. Blocking snail proteins activity has the potential to avert cancer cell metastasis by interfering with such cellular processes as remodelling of the actin cytoskeleton, migration and invasion, which are clearly associated with the aggressive phenotype of the disease. Moreover, the link between factors from the snail family and cancer stem cells suggests that inhibitory agents may also prove their potency as inhibitors of cancer recurrence.

Key words: Snail1, colorectal cancer, calcitriol, VDR, Wnt signalling.

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The role of Snail1 transcription factor in colorectal cancer progression and metastasis

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Introduction

It is generally accepted that a fundamental process for distant metastasis formation is epithelial-mesenchymal transition (EMT), during which tumour cells lose their epithelial properties and acquire a fibroblast-like phenotype. As a consequence, reduced intercellular adhesion, enhanced invasiveness and increased apoptotic resistance of cells have been observed [1, 2]. In developmental and cancer EMT, signals promoting and regulating this process originate from a tissue microenvironment and usually act as transcriptional repressors. Moreover, they are associated with the zinc-finger factors including Snail1, Snail2 (Slug), ZEB1/2 and the basic helix-loop-helix proteins (bHLH) with Twist1/2. Among signalling molecules there are also goosecoid and FOXC2, which repress transcription indirectly via unknown mechanisms [3, 4].

Snail1 as a transcription factor has been shown to be crucial for cellular movement during cancer progression and metastasis. Therefore, in colon cancer patients, enhanced levels of Snail1 are usually associated with poor clinical outcome, probably due to down-regulation of E-cadherin expression [5, 6]. There is evidence showing that the E-cadherin gene (*CDH1*) is also inactivated in diffuse type cancers such as breast lobular carcinoma and diffuse gastric carcinoma. In these cases, neoplastic cells have lost their epithelial properties and display a highly invasive pattern [7, 8]. The question arises regarding which factors are responsible for E-cadherin silencing during EMT. The continuous expression of this epithelial marker during developmental EMT in Snail1-deficient mouse embryos clearly supports the idea that transcriptional repression of E-cadherin is mostly related to Snail1 activity [9].

As mentioned earlier, Snail1 plays a significant role in the development of human malignancies, including those of the gastrointestinal tract. One of these is colorectal cancer, which is one of the most aggressive cancers and a common cause of cancer-related death in westernised countries [10]. The high mortality, accounting for over 90% of colorectal cancer-related deaths, is linked to the ability of cancer cells to spread beyond the large intestine to distant locations [11]. These metastatic abilities of colon cancer cells are closely associated with EMT and transcription factors which are responsible for its activation. Therefore, in this paper we will discuss the role of Snail1 in colorectal cancer progression and metastasis.

Expression of Snail1 in colorectal premalignant and malignant lesions

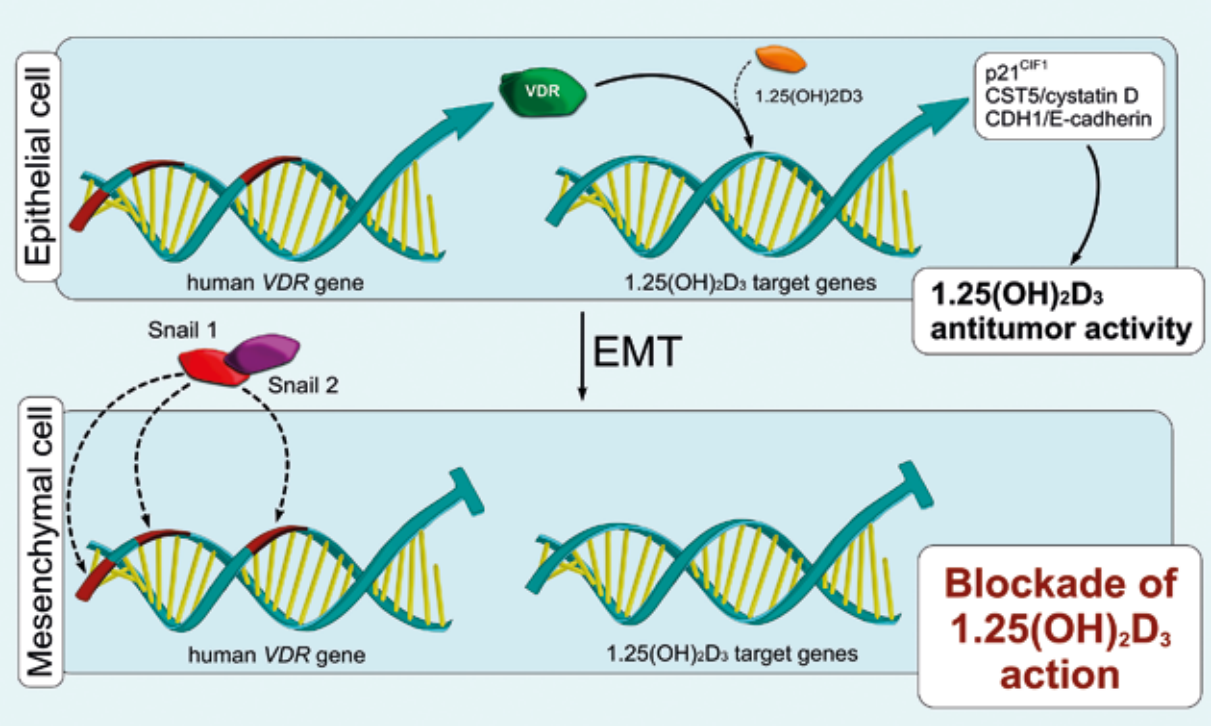
The first stage of colorectal cancer is adenoma. During cancerogenesis this lesion progresses to adenocarcinoma and then to metastatic cancer. Adenoma is also the first stage of colorectal cancerogenesis exhibiting Snail1 expression, which is inversely correlated with expression of E-cadherin [5].

This finding is in agreement with the statement that Snail1 acts as a transcriptional repressor of *CDH1*. It is well demonstrated that repression of *CDH1* is a consequence of molecular reaction cascade, during which a major role is played by interactions with regions containing the consensus core sequence (CAGGTC), a motif that is a subset of E-box (CANNTG) [12, 13]. The expression of Snail1 in adenoma tissue suggests that the EMT process promoting cancer metastasis is already active in precancerous lesions. Studies with the use of transgenic mouse models such as HER-2 and PyMT have revealed that tumour cells may disseminate in morphologically benign hyperplastic lesions [14]. Metastatic mammary epithelial cells have been observed in the lungs of mice carrying the PyMT oncogene at the early stage of breast tumorigenesis when the mammary tree appears to be at normal or at premalignant stage. It would appear that cells acquire invasive abilities even before the process of malignant transformation is completed [15]. Additionally, Röcken suggests that neoplastic cells may spread during the initial steps of cancer development. It is worth noting that the main class of cells, which possess the ability to inhibit the growth of disseminated tumour cells, are $CD8^+$ T cells. They restrain the growth of neoplasias not by cytotoxic effects, but in a cytostatic manner [16]. Eyless *et al.* found that in the presence of $CD8^+$ T cells, the proliferating $Ki67^+$ cells were characterised by a very low level. Moreover, the number of $Ki67^+$ tumour cells grew significantly in the absence of $CD8^+$ T cells. Thus, the cytostatic inhibition of cancer cell growth may be responsible for keeping disseminated tumour cells from spreading to different distant organs [17]. In humans, tumour cell dissemination could take place in morphologically non-invasive lesions as well. For example, in patients with ductal carcinoma in situ (DCIS), cytokeratin-positive disseminated tumour cells have been detected in their bone marrow [18]. Moreover, cytokeratin-positive as well as EpCAM-positive disseminated tumour cells have also been observed in the bone marrow of patients with colorectal adenomas [19]. It should be stressed here, that in the case of colon tissue, Snail1 expression has also been revealed in normal mucosa in close vicinity to the tumour lesions. Interestingly, some differences between non-pathological tissue and cancer tissue have been demonstrated at the subcellular level, where the shift of the protein to the cytoplasm with mixed cytoplasmic and nuclear or pure cytoplasmic expression has been detected [20]. Regarding Snail1 expression in colorectal mucosa with no specific pathological changes, some published results suggest that Snail1 is not connected with malignant transformation only. In normal tissue, this factor may be involved in the maintenance of homeostasis by regulation of cellular proliferation, differentiation and apoptosis [20].

Moreover, up-regulation of Snail1 both at the level of mRNA and protein has been reported to promote regional and distant lymph node metastasis [21, 22]. Snail1 expression as a marker for lymph node metastasis has also been reported in the case of breast cancer and oesophageal squamous carcinoma [22–24]. Interesting results have been obtained by Franci *et al.* Their study showed that about 77% of colon cancer samples display Snail1 immunostaining in the stroma, especially in the stromal cells with fibroblast-like phenotype [25–27]. Expression of this factor in the stroma will indicate how many cells possess the ability to escape from the primary tumour mass, and how many cells may invade the basal lamina to colonise distant target organs [28]. Therefore, the presence of Snail1 immunoreactive cells in the stroma may serve as prognostic marker in patients with colon cancer. It is worth noting that intratumoural injection with Snail1-specific monoclonal antibody inhibits tumour growth and metastasis followed by an increased number of stromal tumour infiltrating lymphocytes (TILs). It is not surprising then, that Snail1 immunoreactivity in tumour stroma is related to distant metastasis ($p = 0.006$) and lower specific survival of patients ($p = 0.011$) [26]. Importantly, this correlation was also detected in stage I and II, which are known to display a variable prognosis. The capability to predict recurrence at these stages would be important from a clinical point of view because markers for them have not yet been identified. Thus, the expression of Snail1 would help to distinguish patients with an unfavourable clinical outcome and would be applicable for chemotherapy, which is not a treatment commonly used in the early stages of colorectal tumours [26]. Although Kroepil *et al.* did not find a prominent correlation between Snail1 expression and E-cadherin loss in cancer tissue, they did observe a significant correlation between the expression of Snail1 in the tumour and Snail1 expression in the corresponding lymph node metastasis. Interestingly, they did not find any evidence of Snail1 expression with clinical parameters such as N-stage, grading, age or sex [29]. It is worth noting that also no association between Snail1 expression and clinical parameters has been revealed in adenocarcinomas of the upper gastrointestinal tract [30]. Probably there are other more powerful mechanisms which are implicated in EMT triggering during colon cancer development, e.g. factors associated with the Wnt signalling pathway. Larriba *et al.* revealed that Wnt signalling and Snail1 may be interconnected by multiple positive loops, possibly adding to the robustness of both [31].

Snail1 expression and anticancer actions of vitamin D analogues

Some published results indicate that vitamin D intake shows a protective effect against colorectal carcinogenesis [32–34]. Also analogues of vitamin D such as 1α,25-dihydroksyvitamin D_3 [calcitrol, $1,25(OH)_2D_3$] demonstrate, additionally to its classical role in regulation of calcium and phosphate homeostasis, an anti-proliferative, pro-apoptotic and pro-differentiation impact on a wide array of cancer cells *in vitro* [35, 36]. Therefore, it is not surprising that vitamin D derivatives with reduced hypercalcaemic activities are under clinical investigation for use against some malignancies, including colon cancer. Nevertheless, only a small population of colon cancer patients respond to this therapy, because of the loss of vitamin D receptor (VDR) expression, which belongs to the nuclear hormone receptor superfamily [37, 38]. A study by Palmer *et al.* demonstrated that human colon cancer cells such as SW480-ADH



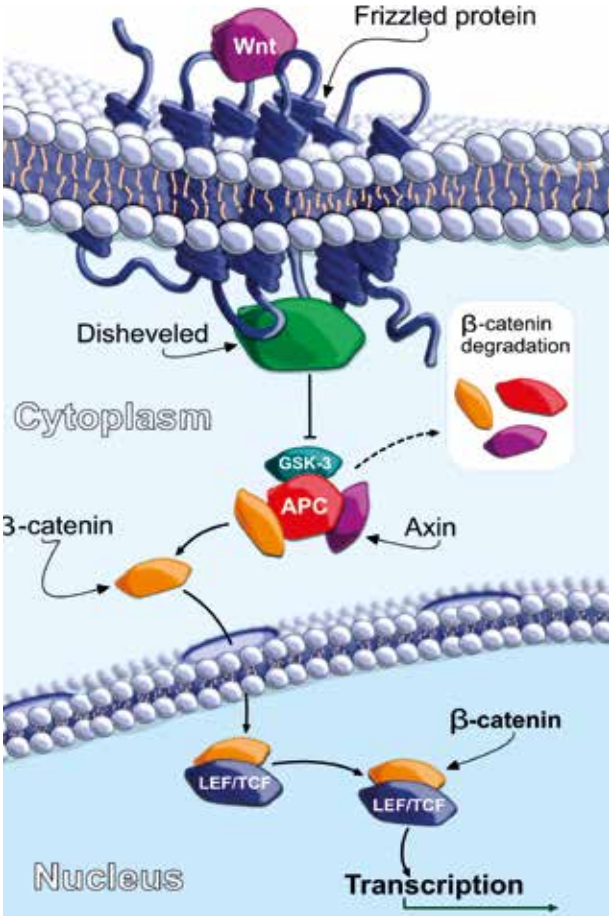
Under physiological circumstances, colon epithelial cells express VDR. Therefore, in such cases they are responsive to calcitrol antitumoral activity. Overexpression of snail factors in cancer cells is thought to be a major "reason" for EMT induction and cancer progression, mostly due to the ability of snail factors to bind to three E-boxes within human VDR gene promoter. Consequently, cells lack their ability to respond to calcitrol antitumor activity that is associated with the inhibition of VDR expression (based on [40]).

Fig. 1. Repression of VDR and calcitrol activity during EMT induced by snail transcription factors

with expression of VDR are sensitive to the anticancer action of calcitrol [39]. In this case, the ligand-activated VDR induces the process of differentiation and expression of E-cadherin. Interestingly, E-cadherin and VDR expression were down-regulated in SW480-ADH and MaCaT cells when they were co-cultured with Snail1-expressing cells [39]. This leads to the question regarding which factors are responsible for the loss of VDR expression during colon cancer progression. Probably, the loss of VDR expression is related to Snail1 up-regulation. Therefore, the high expression of this factor may be responsible for the failure of therapy with vitamin D analogues in patients with colon cancer [37, 38]. Larriba *et al*. revealed that Snail1 and Snail2 bind to the promoter region of exon 1a of the human VDR gene and repress its expression. The repressive effects of Snail1 and Snail2 on VDR gene promoter are similar and are mediated by three E-boxes (CAGGTG/CACCTG), which are present in the first 400 nucleotides of human VDR promoter (Fig. 1). Both Snail1 and Snail2 cooperate to repress VDR promoter, showing an additive effect because the level of VDR expression has is decreased in tumours co-expressing Snail1 and Snail2 compared to those expressing only one of these genes [40, 41]. The association between Snail factors expression and down-regulation of VDR is absent in tumours in which p300 and CtBP (C-terminal binding protein) are strongly expressed. This suggests that the level of co-repressor protein CtBP and nuclear acetylase p300 are critical for Snail1 activity [42].

Cooperation of Snail1 with Wnt signalling pathway

A growing number of studies have revealed that calcitrol and other non-hypercalcaemic analogues suppress cellular proliferation and provoke differentiation in the case of colon cancer [34]. This is probably due to the formation of VDR/β-catenin complexes and subsequent expression of E-cadherin, which take a part in redistribution of β-catenin to the plasma membrane [39]. β-catenin is one of the most significant factors implicated in the canonical Wnt signalling pathway, which plays a major role in colonic epithelial differentiation [43]. In the case of constitutive expression of this pathway, β-catenin is accumulated in the nuclear cell compartment and binds to the proteins of the T-cell factor/lymphoid enhancer factor (TCF/LEF) family (Fig. 2). These nuclear complexes regulate expression of genes involved in proliferation, invasiveness and angiogenesis [44]. In this context, it should be noted that neoplastic cells located at the invasive front of colorectal cancer tissues frequently demonstrate increased levels of nuclear β-catenin. These cells gain a mesenchymal-like phenotype as a result of mutations occurring in adenomatous polyposis coli (APC) or β-catenin genes, which are also the major mechanisms responsible for aberrant activation of Wnt signalling [11, 45, 46]. Therefore, colon cancer cells with an invasive phenotype may show both the aberrant Wnt



The canonical Wnt signalling pathway plays a significant role in the regulation of epithelial cell proliferation, differentiation, adhesion and apoptosis. A great number of studies have revealed that constitutive activation of this pathway is also involved in the EMT process and cancer invasion. During Wnt pathway activity, a pivotal role is played by interactions between β-catenin and members of the TCF/LEF-1 transcription factor family. β-catenin, a prominent mediator of Wnt pathway, exists in the cell membrane and acts as a linking protein of E-cadherin. Binding of Wnt-protein ligand to the Frizzled family receptor passes the signal to the Dishevelled protein (Dvl) inside the cell. This in turn is responsible for inhibition of the destruction complex composed of three different proteins, such as glycogen synthase kinase 3β (GSK-3β), axin and adenomatous polyposis coli (APC). This inhibition leads to the stabilisation of β-catenin and promotes its accumulation in the cytoplasm followed by translocation to the nucleus. In the nucleus, β-catenin forms transcription complexes with TCF, which influence the expression of various target genes such as cyclin D1 and matrix metalloproteinases (MMPs) that are involved in the regulation of cellular events important for cancer invasion.

Fig. 2. The canonical Wnt signaling pathway

signalling and expression of EMT-activating transcription factors such as Snail1.

As indicated earlier, both Snail1 and Snail2 are responsible for the low level of VDR receptors in colon cancer cells. Re-expression of this receptor may restore the inhibitory effect of calcitrol associated with the formation of VDR/β-catenin complexes interfering with those of β-catenin/TCF. These molecular events are responsible for the partial inhibition of Wnt signalling in colon cancer cells. But for the completed inhibition of this pathway, stable redistribution of β-catenin from the nucleus to the plasma membrane is required. Such redistribution is associated with induction of E-cadherin expression, and, therefore, the poorer induction of E-cadherin in Snail1 + VDR cells is observed [47]. Importantly, this weaker E-cadherin expression activated by calcitrol is related to repression of CDH1 promoter mediated by Snail1 [31, 40].

A growing number of studies have demonstrated that tumour-associated macrophages (TAMs) provide soluble factors promoting the growth and metastasis of colon cancer cells [48, 49]. For example, macrophage-derived IL-1β promotes Wnt signalling in colon cancer cells and therefore regulates their growth and survival [50, 51]. Kaler *et al.* revealed that macrophage-induced activation of Wnt signalling in colon cancer cells not only initiates a plethora of cell-intrinsic changes in epithelial cells but also influences communication between epithelial cells and macrophages. Inactivation of the MT β-catenin allele in HCT116 cells prominently changes their interactions with macrophages, which fail to promote Wnt signalling or protect HCT116WT cells from TRAIL-induced apoptosis [52]. FGF19 has also been demonstrated to alter Wnt signalling in HCT116 cells [53]. This factor enhances β-catenin/TCF transcriptional activity both in parental HCT116 cells and in HCT116 cells retaining the WT β-catenin allele [53]. It seems that activation of pathways such as Wnt not only triggers changes in cell structure but also induces stromal modifications, which are needed for cancer development.

Macrophage-derived factors are also associated with stabilisation of Snail1 transcription factor in colon cancer cells [51]. In contrast to the parental HCT116 cells, macrophage-derived factors fail to stabilize Snail1 in HCT116 cells with deleted MT β-catenin allele. The ability of macrophages and IL-1β to suppress TRAIL-induced apoptosis and to generate clonogenic growth of tumour cells is also inhibited in tumour cells with silenced Snail1 expression [51]. More so, active Wnt signalling in colon cancer cells and Wnt-dependent stabilization of Snail1 are needed for tumour cells to provoke macrophages to produce IL-1β. This suggests that macrophage-derived factors protect colon cancer cells from TRAIL (TNF-related apoptosis-inducing ligand)-induced apoptosis through stabilisation of Snail1 [51]. Therefore, the lack of Snail1 stabilisation in HCT116 cells with inactivated MT β-catenin allele underlies the inability of macrophages to protect these cells from TRAIL-induced apoptosis. The results of these studies clearly confirm that induction of Wnt signalling and subsequent stabilisation of Snail1 in colon cancer cells influence their interactions with macrophages, and indicate a fundamental role of Snail1 in the crosstalk between colon cancer cells and macrophages.

Some published results indicate that human colon tumours exhibit heterogeneous levels of Wnt activity [54]. Moreover, cells which are characterised by high levels of Wnt signalling display colon cancer stem cell (CSC) features [55, 56]. Silencing β-catenin in HCT116 cells has been shown to reduce the number of colonospheres which have a great number of CSCs [57]. Macrophages and IL-1β-enhanced Wnt signalling and stabilised Snail1 in tumour cells have been demonstrated to equip cells with stem cell-like characteristics, suggesting that macrophage-derived factors contribute to heterogeneity of colon tumours by

broadening the population of cells which possess cancer stem cell features [51, 58].

In summary, Snail proteins, as a major factor responsible for EMT induction, are promising targets for development of pharmaceutical agents. Blocking snail protein activity has the potential to avert cancer cell metastasis by interfering with such cellular processes as remodelling of the actin cytoskeleton, migration and invasion, which are clearly associated with the aggressive phenotype of the disease. Furthermore, the link between snail and cancer stem cells suggests that inhibitory agents may prove potent as inhibitors of cancer recurrence. Some recent studies revealed that Snail1 might modulate several steps in RNA maturation including polyadenylation, splicing and translation [59]. Also, this factor may regulate gene expression post-transcriptionally by modulation of protein levels involved in pre-mRNA processing and location [59]. It is also worth noting that, additionally to their anti-cancer potential, inhibitors of Snail1 activity would be significant tools to simplify the study of these transcriptional factors in model cell types associated with embryonic development such as neural crests. Nevertheless, further investigations associated with Snail1 are needed, especially for clinical oncology.

The authors declare no conflict of interest.

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