Differential expression of Snail1 transcription factor and Snail1-related genes in alveolar and embryonal rhabdomyosarcoma subtypes

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Abstract: Rhabdomyosarcoma (RMS) represents the most common sarcoma of soft tissue among children. Two main RMS subtypes are alveolar (ARMS) and embryonal (ERMS). The major goal of this study was to find differentially expressed genes between RMS subtypes that could explain higher metastatic potential in ARMS and would be useful for the differential diagnosis. Using RQ-PCR analysis we compared expression of Snail1 and Snail-related genes among 7 ARMS and 8 ERMS patients' samples obtained from the primary tumors and among 2 alveolar and 2 embryonal cell lines. Our results show that Snail1 is highly expressed both in ARMS patients' samples and the alveolar cell lines. We also found that the expression of E-Cadherin was downregulated and the expression of Matrix Metalloproteinases 2 and 9 (MMP-2 and MMP-9) was upregulated in ARMS. We assume that, as in many tumors, also in RMS Snail1 acts as a regulator for pathways known for their role in cells' metastasis and that Snail1 activity results in increased MMPs and decreased E-Cadherin expression. Our findings may explain higher ARMS aggressiveness. Moreover, we suggest that further studies should be performed to verify if Snail1 can be considered as a potential target for ARMS therapy.

Key words: Snail1, rhabdomyosarcoma, ARMS, ERMS, E-Cadherin

Introduction

Rhabdomyosarcoma. Rhabdomyosarcoma (RMS) usually develops between the second and the fifth year of life and in adolescence. There are three subtypes of RMS. Two main are embryonal (ERMS) and alveolar (ARMS). The third one, uncommon among children, is pleomorphic. The two first subtypes constitute around a half of the soft tissue sarcomas among children and young adults [1]. There are important differences between ARMS and ERMS in clinical and histological picture on which the diagnosis is based. ARMS tumors have, in general, worse prognosis, and occur in older children and teenagers. Common locations for ARMS tumors are: extremities and trunk whereas for ERMS: head and neck and urogenital system [1]. Typical for

part of ERMS tumors is loss of heterozygosity (LOH) of 11p15. It causes Insulin-like growth factor 2 (IGF-2) overexpression and lack of H19- suppressor gen. In around 80% of ARMS tumors translocation t(2;13)(q37;q14) or t(1;13)(p36;q14) leads to Pax3-FKHR or Pax7-FKHR fusion genes formation [2].

Snail 1 and epithelial-mesenchymal transition. Snail1 belongs to the group of conservative zinc finger transcription factors [3]. There are numerous processes on which Snail has a noticeable impact: cell proliferation, apoptosis and survival, cell cycle regulation, cell shape, movement, differentiation, gastrulation or mesoderm formation [3,4]. Particular role of Snail1 has been described in the epithelial-mesenchymal transition (EMT) – the main mechanism responsible for the invasiveness and metastasis of neoplasm in the advanced stages. EMT can be modified by numerous growth factors such as fibroblast growth factor (FGF), hepatocyte growth factor (HGF), transforming growth factor β (TGFβ) [4] or by Notch pathway [5]. It has

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Table 1. Description of RMS cell lines.

Cell line	RMS subtype	Established from	Fusion genes	Literature
SMS-CTR	ERMS	Relapse	no	11
RD	ERMS	Relapse	no	11
RH18	ARMS or ERMS	Primary pelvic tumor	Pax3-FKHR or no	12, 13
RH30	ARMS	Bone marrow metastasis	Pax3-FKHR	14

been suggested that most of these factors modify EMT through the genes belonging to the Snail family, *e.g.* by decreasing the expression of E-Cadherin [6]. Snail activation can also result in an increased expression of MMPs. Activity of Snail1 remains under control of glycogen synthase kinase 3 (GSK3β) – protein functioning as a part of β-Catenin degradation complex [7].

Factors considered to play a role in rhabdomyosarcoma metastatic behavior. The knowledge about the molecular differences between ARMS and ERMS is not complete. Different scientific groups proved the special role of fusion proteins Pax3(or 7)-FKHR and HGF-MET or chemokine pathways in higher ARMS metastatic behavior. The silencing of Pax3-FKHR reduces MET level. What is more, the induction of Pax3-FKHR in the embryonal cell line results in higher CXCR4 and MMP-2 expression [8, 9]. The level of MMP-1, MMP-2 and MMP-9 expression has been shown to be higher in ARMS in immunohistochemical staining with no differences in the level of expression of Tissue inhibitors of Metalloproteinases (TIMPs) [10]. The way in which all these factors interact and the common point of this interaction have not been described.

In our studies we checked the expression of Snail-related EMT- modulating genes. The results indicate different expression of these genes between RMS subtypes, which can modify the metastatic behavior of these subtypes.

Materials and Methods

Cell lines. The human rhabdomyosarcoma embryonal: SMS-CTR and RD and alveolar: RH18 and RH30 subtype cell lines were generously provided by prof. M. Z. Ratajczak (University of Louisville, KY, USA) and were grown at 37°C in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco BRL, Grand Island, New York, USA) and supplemented with antibiotics, L-glutamine and 10% fetal bovine serum (Gibco). Cell lines are characterized in Table 1.

Material from patients. Paraffin embedded tissues contained samples of 15 RMS primary tumors. First group contained 7 patients with ARMS and the second 8 patients with ERMS. The patients were diagnosed in Polish-American Institute of Pediatrics in Cracow and selected according to the diagnosis of a primary tumor, the availability of material (paraffin embedded tissues), undoubted classification to histological subtype and the quality of isolated RNA.

Extraction of RNA and reverse transcription reaction. RNA from cell lines was isolated using RNeasy Mini Kit (Qiagen, Valencia, California, USA). Paraffin embedded tissues were cut into 10 μm-thick sections, then deparaffinized in xylene and rehydrated through a graded ethanol series. Tissues were digested with Proteinase K, then treated with TRIzol (Invitrogen, Carlsbad, California,USA). RNA was isolated using modified acid guanidinium thiocyanate-phenol-chloroform method [15]. cDNA was obtained from RNA by performing RT-PCR with random primers (Promega, Madison, Wisconsin, USA.) and Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, California,USA).

Real-time PCR. The expression of following genes in cell lines (50ng of cDNA/well) and material from patients (120 ng of cDNA/well due to lower quality of material received from paraffin-embedded tissues than from cell cultures) was estimated during real-time PCR (Applied Biosystems, Foster City, California, USA): Snail1 (Hs00195591_m1), Slug (Hs00161904_m1), Twist (Hs00361186_m1), β-Catenin (Hs99999168_m1), E-Cadherin (Hs00170423_m1), N-Cadherin (Hs00169953_m1), Met (Hs01565589_m1), Mmp-2 (Hs00234579_m1), Mmp-9 (Hs00234579_m1). The expression of HGF (Hs00300159_m1), Timp-1 (Hs00171558_m1) and Timp-2 (Hs00234278_m1) was checked only in the cell lines. Also the expression of the following genes (sequence of primers) was checked: Pax3-Fkhr (F 5'AAC CCC ACC ATT GGC AAT G, R 5'ACC CTC TGG ATT GAG CAT CCA) and Pax7-Fkfr (F 5'TTT GAG ACC CAC TAC CC, R 5'ACC CTC TGG ATT GAG CAT CCA). The control for probes was GAPDH (Hs99999905_m1) and the control for primers was β-Actin (F 5'GGA TGC AGA AGG AGA TCA CTG, R 5'CGA TCC ACA CGG AGT ACT TG). The reactions were performed in ABI PRISM 7300 Sequence Detection System (Applied Biosystems) with the use of TaqMAN PCR Master Mix (Applied Biosystems). The reactions with primers were performed with the use of Sybr Green PCR Master Mix (Applied Biosystems).$2^{-\Delta\Delta CT}$ method allowed calculating relative expression of genes.

Western blot. Total protein was isolated from cells using M-PER buffer (Pierce, Rockford, Illinois, USA) with proteases and phosphatases inhibitors (Sigma, St Louis, Missouri, USA). Nuclear fraction was isolated using NEB-B buffer (29 mM HEPES pH 7.9; 0.4 M NaCl; 1 mM EDTA; 1 mM EGTA). Protein concentration was measured with EL800 detector and Protein Assay (Bio-Rad, Hercules, California, USA). Then electrophoresis on a sodium dodecyl sulfate-polyacrylamide gel (6 and 12%) and transfer of proteins to polyvinylidene diflouride membrane (BioRad) was performed. Blot was incubated with 1% BSA for 1 hour and then overnight with primary antibodies: anti-SNAI1 1:1300 Sc-28199 (Santa Cruz Biotech., Santa Cruz, California, USA), anti-MET 1:1000 Sc-161 (Santa Cruz Biotech.) and GAPDH as a control 1:10000# 2118 (Cell Signaling, Danvers, Massachusetts, USA). Secondary 1:5000 anti-rabbit IgG, antibody Sc-2054 (Santa Cruz Biotechnology) was conjugated with horseradish peroxidase. Proteins were visualized on
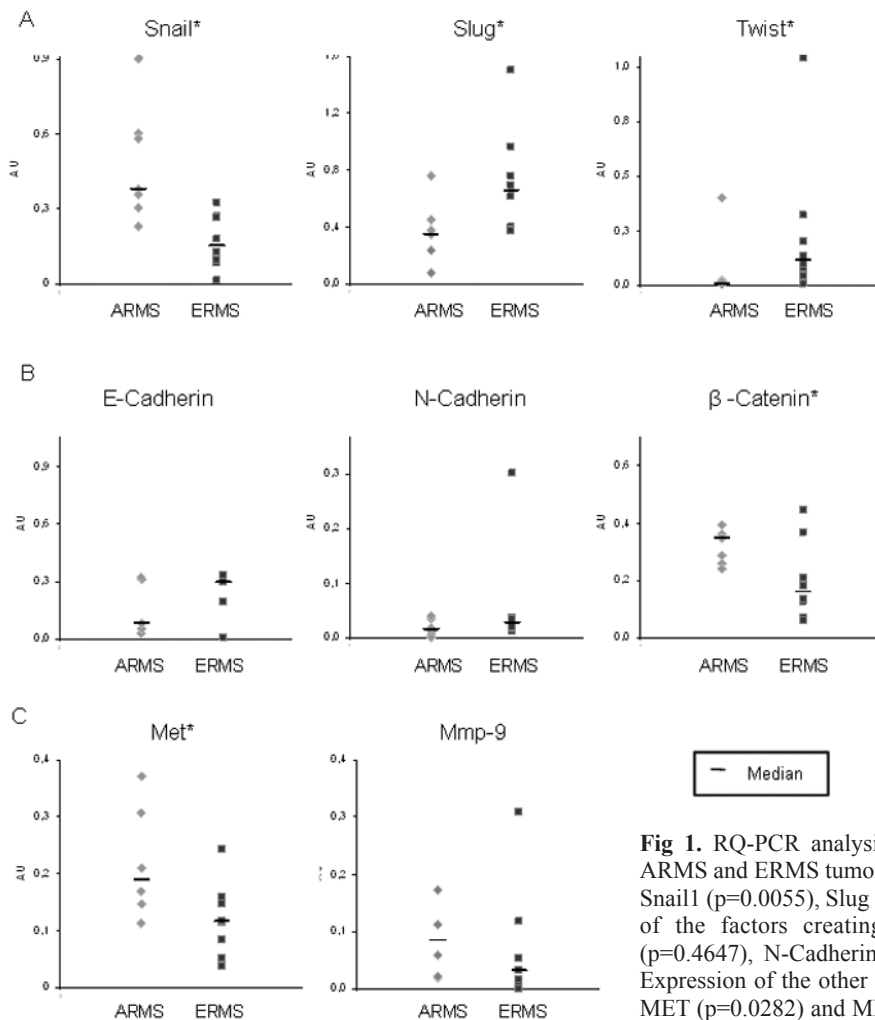
Fig 1. RQ-PCR analysis of mRNA in material from patients with ARMS and ERMS tumors. **(A)** Expression of the transcription factors: Snail1 (p=0.0055), Slug (p=0.0372), Twist (p=0.0491). **(B)** Expression of the factors creating Cadherin-Catenin junctions: E-Cadherin (p=0.4647), N-Cadherin (p=0.1324) and β-Catenin (p=0.0491). **(C)** Expression of the other factors involved in the process of metastasis: MET (p=0.0282) and MMP-9 (p=0.2531), *p<0.05.

photographic film (Amersham Life, Sciences, Little Chalfont, UK) using chemiluminescent reagent (Amersham Life).

Statistical analysis. Experiments for cell lines were repeated 2-4 times. In order to estimate the statistical difference between patients Mann Whitney U-test and between cell lines nonpaired T-Student test were applied. Correlations between genes were checked by fast regression analysis in Statistica 6.1 package.
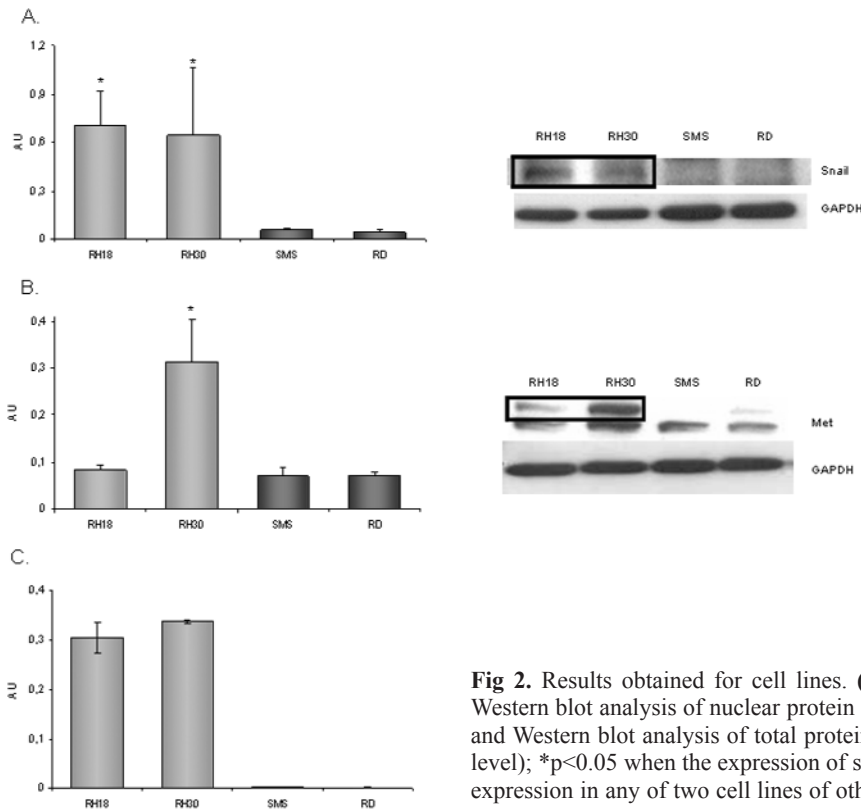
Results

The expression of EMT modulating genes in rhabdomyosarcoma subtypes

Higher expression level of Snail1 mRNA was detected in ARMS in comparison to ERMS subtype (Fig. 1A). These results were confirmed using established RMS cell lines. RH30 and RH18 alveolar cell lines had higher expression of Snail1 mRNA and protein level when compared to SMS-CTR and RD embryonal cell lines (Fig. 2A). In contrary the expression of Slug and Twist transcription factors was higher in ERMS tumors (Fig. 1A).

Different expression of Snail1-related genes in rhabdomyosarcoma subtypes.

Cadherins and MMPs are direct targets for Snail1. Thus we checked the expression of E- and N- Cad-herins together with the expression of MMP-2 and MMP-9 in the patients' samples. The expression of both E-Cadherin and N-Cadherin was lower in ARMS in comparison to ERMS (Fig. 1B), but these tendencies were not statistically significant. Similar results were obtained for the cell lines (Fig. 3A higher expression of E-Cadherin in SMS cell line; Fig. 3B higher expression of N-Cadherin in both ERMS cell lines). We did not find any differences in MMP-2 expression in the primary tumors samples (data not shown). However, RH30 ARMS cell line expressed MMP-2 at high level (Fig. 3C). The expression level of MMP-9 was higher in ARMS tumor samples (Fig. 1C). MMP-9 was also high-ly expressed in RH18 ARMS cell line (Fig. 3D). No dif-ferences were visible in case of TIMPs (data not shown).

Next, we compared the expression of other genes involved in the metastatic process. β-Catenin expression

Fig 2. Results obtained for cell lines. **(A)** Snail1 (RQ-PCR analysis of mRNA and Western blot analysis of nuclear protein level). **(B)** MET (RQ-PCR analysis of mRNA and Western blot analysis of total protein level). **(C)** Pax3-FKHR (analysis of mRNA level); *p<0.05 when the expression of studied gene in this cell line is compared to the expression in any of two cell lines of other subtype.
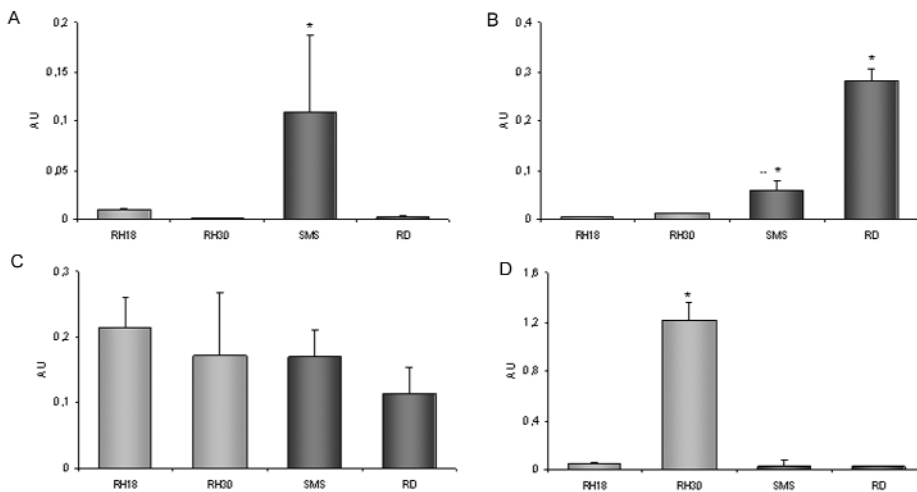

Fig 3. Results obtained for cell lines. **(A)** E-Cadherin; **(B)** N-Cadherin; **(C)** MMP-9; **(D)** MMP-2; (RQ-PCR analysis of mRNA). *p<0.05 when the expression of studied gene in this cell line is compared to the expression in any of two cell lines of other subtype.

was noticeably higher in the tumor samples of ARMS subtype (Fig. 1B). We also confirmed the earlier reports that MET receptor expression is higher in ARMS than in ERMS tumors (Fig. 1C). This was again confirmed using RMS cell lines (Fig. 2B – higher expression on mRNA level in RH30 and on protein level in both alveolar cell lines). We did not see any differences in the expression of HGF (data not shown). This is probably due to very low expression of HGF in RMS (only detected using real time RT-PCR but not regular RT-PCR) that makes the interpretation difficult.

According to our results RH18 and RH30 cell lines have high expression of Pax3-FKHR on mRNA level, which is typical of fusion-positive ARMS (Fig. 2C).

Correlation between the expression of Snail and the other genes

The scrutiny of the data obtained for the tumor samples revealed also some existing correlations between the expression of Snail and the other genes. The expression of this transcription factor was positively
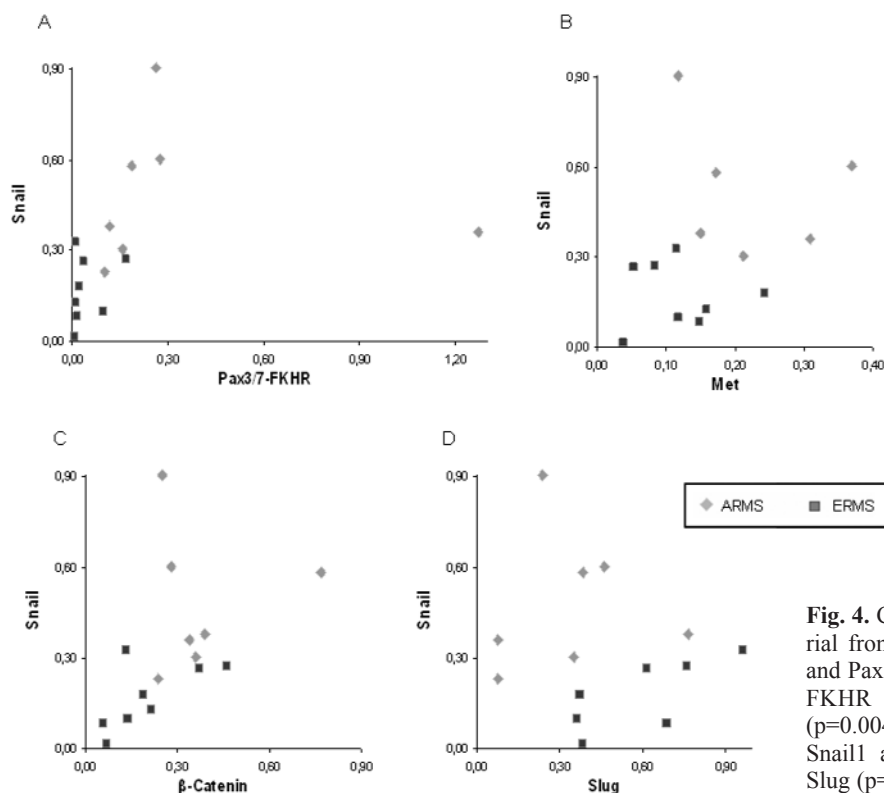
Fig. 4. Correlations between genes obtained in material from patients by RQ-PCR analysis. **(A)** Snail1 and Pax3/7-FKHR (a sum of Pax3-FKHR and Pax7-FKHR expression received for each patient) (p=0.0045); **(B)** Snail1 and MET (p=0.0019); **(C)** Snail1 and β-Catenin (p=0.0045); **(D)** Snail1 and Slug (p=0.0045).

correlated with Pax3(or 7)-FKHR (Fig. 4A) and MET receptor (Fig. 4B) expression. These proteins are well known for their role in the augmenting the metastatic behavior of ARMS tumors. Positive correlations were also seen between Snail1 and MMP-9 (data not shown) and β-Catenin (Fig. 4C). We observed a negative correlation between Snail and Slug (Fig. 4D).

Discussion

In this study we have observed the distinct expression of Snail1 transcription factor and its related genes between rhabdomyosarcoma subtypes. The expression of Snail1 positively correlated with the expression of Pax3(or 7)-FKHR fusion gene. This correlation can be a result of a direct regulation of Snail1 expression by the fusion genes or an indirect through other proteins such as MET, which has been shown to play a role in regulating Snail1 expression in other tumors [16] through phosphorylation of GSK3β and its inactivation. Thus, higher expression of Snail1 might be explained by higher expression of MET in alveolar subtype (in RH30 cell line on mRNA level, in both alveolar cell lines on protein level and in patients samples). Interestingly, in case of RH18 alveolar cell line the expression of Snail1 is high and Pax3-FKHR fusion gene is present, but the mRNA level of MET is similar to this in embryonal cell lines. However, the level of

MET protein expression is similar to one observed for RH30 cells and this might again explain higher expression of Snail in this cell line. The lack of significant differences in the expression of HGF between ARMS and ERMS is also unexpected (data checked only in cell lines due to small amount of RNA received from tumor samples). One of the possible explanation is that highly expressed MET receptor might be activated not only in a result of HGF stimulation.

Approximately 20% of ARMS tumors do not possess the translocation [1]. Thus, expression of other genes could be responsible for highly metastatic phenotype of ARMS. We showed here that the expression of Snail1 is higher in ARMS in comparison to ERMS patients' samples and this finding was confirmed using established cell lines.

We noticed that in ARMS tumors the expression of E-Cadherin is lower in comparison to ERMS and negatively correlates with the expression of Snail1. High E-Cadherin level is also noticed in SMS (but, surprisingly, not in RD) embryonal cell line. The decreased expression of E-Cadherin could be responsible for malfunction of cell-cell junctions in ARMS and higher ability of these tumors to leave the tumor bed and metastasize. We also noticed lower expression of N-Cadherin in ARMS. Since, the expression of E-cadherin is also regulated by other members of Snail-family *e.g.* Slug [17], it is possible that low E-cadherin
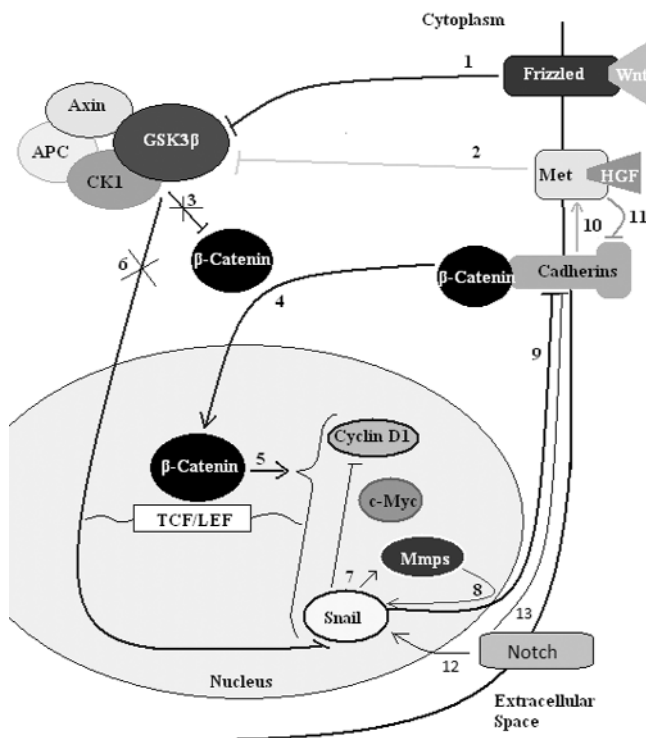
Fig. 5. Presumable interactions between factors responsible for ARMS higher metastatic behavior. Activation of Frizzled receptors by Wnts and MET receptor by HGF leads to phosphorylation of GSK3β protein [21,24] and, the same, inactivation of β-Catenin degradation complex (1, 2). β-Catenin, freed from destructive influence of its degradation complex (3), is transported to the nucleus (4), where, acting through TCF and LEF nuclear proteins it can work as a transcription factor. High nuclear level of β-Catenin results in increased expression of its target genes *e.g.* Snail1 [25], MMPs, MET and Cyclin D1 (5). Phosphorylated form of GSK3β cannot inhibit Snail, which additionally promotes Snail1 activity (6). Snail1 itself increases the expression of other genes e.g. MMPs and inhibits Cyclin D1 [26] (7). Some of MMPs induce feedback activation of Snail1 (8). Snail represses Cadherins expression what frees β-Catenin from its associations with Cadherins and leads to MET internalization (9, 10) (as the presence of MET in cell membrane protein fraction depends on Cadherins presence [27]). Also MET has the ability of directing Cadherins to degradation pathway (11) [28]. The expression of Snail is also directly and indirectly up-regulated by Notch pathway (12), which was shown to play the crucial role in EMT process. Also E-Cadherin down-regulation requires Notch signaling (13) [5].

expression in RD cells is due to high expression of Slug. In case of RD embryonal cell line low E-Cadherin level may not indicate higher metastatic potential (similar to ARMS) because there is a high level of N-Cadherin detected in this cell line. N-Cadherin is responsible for creating normal cell junctions in muscular tissue [18]. Higher expression of N-Cadherin in ERMS subtype needs a further analysis.

MMPs were proved to be regulated by Snail1 [19]. In our study we have shown that MMP-9 expression was higher in ARMS primary tumors and in RH18 cell line (established from primary change). Moreover, the expression of MMP-9 positively correlated with the expression of Snail1. RH30 cell line established from metastatic lesion had significantly higher expression of MMP-2 in comparison to other cell lines. There were no significant differences in the expression of MMP-2 between ARMS and ERMS primary tumors what may suggest important role of this gelatinase in aggressive late stage ARMS. Higher level of MMP-2 has already been attributed to ARMS subtype [19,20].

β-Catenin was shown to interplay with Snail1 in EMT facilitation [16,21]. In our research we have observed higher expression of β-Catenin in case of ARMS primary tumors. However, we have not observed any differences in β-Catenin expression in RMS cell lines. This discrepancy can result from the fact that SMS and RD cell lines were established from relapses where Wnt-β-Catenin pathway can play more significant role than in the primary ERMS tumors. Existing data suggest that the concurrent inhibition of Wnt-β-Catenin and HGF-MET axis decreases Snail1 expression and restrains the growth of the soft tissue sarcomas [22,23]. We imply that the inhibition of Snail1 itself, as a factor that links these pathways (and presumably other pathways and factors such as: Pax3(or 7)-FKHR, chemokine axis, MMPs), would intensify this effect (Fig. 5).

Other proteins proved to play an important role in EMT (i.e. by inhibiting E-Cadherin transcription) are Twist and Slug. Data regarding RMS tumors, in general, show high expression of Twist [29]. Twist inhibits factors responsible for miogenesis, such as MyoD [30] and increases N-Cadherin expression [31]. In our study the expression of Twist and Slug was lower in ARMS than in ERMS primary tumors. Using established cell lines, however, we did not confirm these results. LOH characteristic for many ERMS tumors has also been related to cell differentiation disorders. It has been shown that expression of Twist is higher among patients with LOH 11p15 than in other patients [32]. Expression of IGF-2 resulted from this LOH was also shown to influence the level of Slug [33].

In this paper we have shown for the first time that the distinction between RMS subtypes could be made on the base of the expression of Snail1 transcription factor. The expression of Snail1 is higher in alveolar subtype in comparison to embryonal in the patients' samples and this finding was confirmed using established ARMS and ERMS cell lines both on RNA and protein level.

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