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Relevance of targeting *RET/PTC* junction oncogene and Wnt/β-catenin pathway in the treatment of papillary thyroid carcinoma: skill of 8-year work

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Papillary thyroid carcinoma (PTC) is the most common endocrine gland malignancy and occurs frequently due to the radiation exposure. PTC is characterized by the paracentric inversion in chromosome 10 leading to the fusion of RET with several genes present in thyroid named PTC. The RET/PTCs junction oncogenes are present in around 80 % of papillary thyroid carcinoma, the most frequent ones are RET/PTC1 and RET/PTC3. Interestingly, RET/PTCs are found only in the tumour cells and not in the surrounding normal tissues, therefore, they represent a good target for RNA interference strategies. We aimed, on the one hand, to inhibit dedifferentiation due to the RET/PTC junction oncogene by siRNA and, on the other hand, to investigate a role of Wnt/β-catenin pathway in the regulation of a tissue-specific transcription factor, the thyroid transcription factor-1 (TTF-1) essential for the differentiation of the thyroid. In this paper we summarised our main results obtained during eight years that pointed a new therapeutic strategy for papillary thyroid carcinoma.

Keywords: papillary thyroid carcinoma, RET/PTCs junction oncogenes, siRNA, tumour cells.

Introduction. Junction oncogenes are the consequence of genomic rearrangements, especially translocations, leading to the intragenic gene fusion found in around 20 % of total cancers. First, they were described in chronic myeloid leukemia where a translocation between chromosome 9 and 21 t(9;22)(q34;q11) occurs leading to the junction of *brc-abl* genes. Nowadays, they are well described in sarcoma (Ewing sarcoma) and in carcinomas such as papillary thyroid carcinoma (PTC). In fact, thyroid carcinomas are the most common endocrine gland malignancy that can be classified according to their histopathological characteristic [1]. Rearrangement of the *RET* gene, also known as *RET/PTC* rearrangement, is the most common genetic alteration identified to date in thyroid papillary carcinomas. The possibility to inhibit the expression of an oncogene at the mRNA level, instead of blocking the function of the gene product, has elicited for a long time a great interest as the potential therapeutic applications are obvious. Therefore, RNA interference offers promising new opportunities to target very specifically the genes deregulated in PTC cancers carrying the *RET/PTC* junction oncogenes.

It is well known that cancer cell transformation by the expression of an oncogene may act in two different ways, which can be simultaneous. First, the oncogene is able to enhance the cell growth by affecting different proteins involved in cell proliferation; second, the oncogene blocks the cell death. In thyroid carcinoma, one of the proteins involved in the cell proliferation is the thyroid transcription factor-1 (TTF-1). TTF-1 is only expressed in the thyroid follicular cells and together with Paired box gene-8 (PAX-8) controls the expression of thyroglobulin (Tg), thyroperoxydase (TPO), thyrotropin receptor (TSH) and the sodium/iodide symporter (NIS), calcitonin and major histocompatibility complex class I genes in the thyroid [2–4]. In this way, the combination of these two factors plays a role in the expression of the thyroid-specific phenotype.

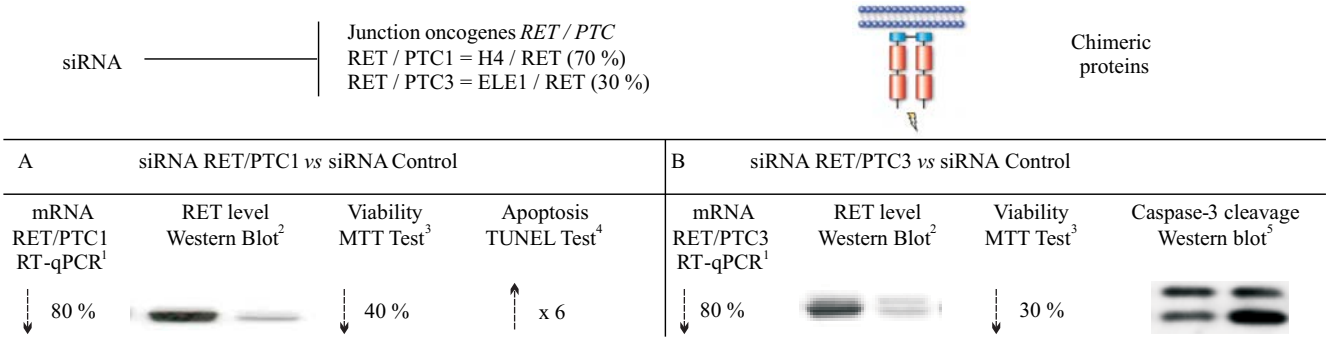

Fig 1. Summary of the main results obtained *in vitro* after knocking-down by siRNA RET/PTC junction oncogene. Cell lines expressing RET/PTC1 (*A*) or RET/PTC3 (*B*) were transfected with 50 nM of siRNA CT, siRNA RET/PTC1 or siRNA RET/PTC3. A^1 and B^1 – the expression of RET/PTC1 or RET/PTC3 mRNA levels was analyzed by real time RT-PCR (RT-qPCR) and recorded as % of modification of treated cells with siRNA RET/PTC1 or RET/PTC3 compared to the siRNA control. A^2 and B^2 – RET protein level in cells transfected with siRNA control (left bands) and transfected with siRNA RET/PTC1 or siRNA RET/PTC3 (right bands) at 50 nM. Proteins were extracted and analyzed by Western blot using Ret antibody. A^3 and B^3 – the viability of cells expressing RET/PTC1 (*A*) or RET/PTC3 (*B*) was evaluated by MTT assay after 72 h incubation with therapeutic siRNAs (siRNA RET/PTC1 or siRNA RET/PTC3) or siRNA control at 50 nM concentration. The number of viable cells was measured and 100 % cell viability corresponds to the number of living cells incubated with transfecting agent only. A decrease of cell viability was observed in transfected cells with siRNA therapeutic siRNAs compared to those transfected with the siRNA control. A^4 – apoptosis was evaluated with an *in situ* TUNEL method. To determine the apoptotic index one thousand cells were counted on each slide and the average number of apoptotic cells was established. A 6-fold increase was observed when cells were transfected with the siRNA RET/PTC1 compared to the siRNA control, this testifying apoptosis. B^5 – Western blot of cleaved caspase-3 was performed 72 h after RP3 cells (expressing *RET/PTC3* junction oncogene) transfected with siRNA RET/PTC3 or siRNA control at 50 nM. Increase of cleaved caspase-3 is clearly observed once cells were transfected with siRNA RET/PTC3, this also testified apoptosis

Since 2008 our aim was to offer a personalized treatment for cancer patients with junction oncogenes by: i) restoring differentiation by knocking down junction oncogenes using small interference RNA strategy, and ii) maintaining the expression of genes involved in thyroid differentiation. In the following paragraphs the strategies that we followed to reach our goal will be described and the biological relevance of the results obtained during the last years will be discussed.

***RET/PTC* junction oncogenes and papillary thyroid carcinomas (PTC)**. Papillary thyroid carcinomas are characterized by gene rearrangements affecting the *RET* (rearranged during transfection) proto-oncogene, which is located on chromosome 10q11.2 and codes for a cell membrane tyrosine kinase receptor [5]. This gene plays a role in the regulation of cell growth, survival, differentiation and migration [6]. In PTC, *RET* fuses with different ubiquitous genes to give various *RET/PTC* fusion rearrangements, that results in an abnormal expression of the chimeric RET protein which is constitutively activated in follicular cells [7]. To date, 13 different fusion patterns have been reported between the *RET* and genes located on different chromosomes [8]. *RET/PTC1* and *RET/PTC3* are the major variants, whereas the others are very rare and have little clinical significance. *RET/PTC1* results from the fusion of *RET* with *H4* gene (also known as *CCDC6*), and *RET/PTC3* arises from *RET* fusion with *ELE1* gene also designated as nuclear receptor co-activator 4; *NCOA4*, *RFG* or *ARA70* [9]. The spatial proximity of *RET* gene with *CCDC6* (10q21) or *ELE1* (10q11.2) during thyrocyte interphase explains the *RET/PTC1* or *RET/PTC3* formation [10].

The prognosis of PTC is generally good, depending on the biological behaviour of the tumour and appropriate initial treatment [11, 12] which includes total thyroidectomy and functional lymph node dissection, followed by radioiodine therapy and rarely, radiotherapy or chemotherapy.

However, a considerable number of patients, approximately 30 %, as shown after 30 years of follow-up, have recurrent disease. This constitutes an area of important research on emerging therapies such as using small interfering RNA (siRNA) to target the *RET/PTC* fusion oncogene which is present only in the tumour cells and not in the surrounding normal cells.

In order to introduce a new pharmacological approach with siRNA against the *RET/PTC1* and *RET/PTC3* junction oncogenes we first designed siRNAs within the junction sequence according to the «method of

scoring» developed by Reynolds *et al*. [13]. Four siRNAs were designed for each sequence. The most efficient one that gave more than 80 % of mRNA inhibition followed by a drastic decrease in the protein content was eligible for further studies. Moreover, siRNA RET/ PTC1 or siRNA RET/PTC3 are able to inhibit only their own junction oncogene showing that a specific fusion sequence is required to target the junction oncogene. From a biological point of view, both siRNA RET/PTC (1 and 3) showed significant inhibitory effects on cell viability and on invasion/migration along with blockage of the cell cycle at G_0/G_1 phase. Additionally, we observed apoptosis induction with caspase-3 and PARP1 cleavage [14, 15]. Fig. 1 summarizes the main results obtained.

However, *in vivo* delivery of siRNA is a key challenge because the biological efficacy of siRNAs is hampered by their short plasmatic half-life due to a poor stability in the biological fluids and by their low intracellular penetration due to their highly hydrophilic character. So far, a wide variety of approaches have been employed to deliver siRNA *in vivo* including viral-vector based and non-viral delivery systems, such as liposomes, nanoparticles, lipophilic conjugates, polymers and cell penetrating peptides [16], however, the safety of these vectors is questionable [17]. Therefore, recently we have conceived a new strategy to deliver siRNAs, based on their conjugation to squalene (SQ), a natural and nonionic biocompatible lipid [18]. This concept termed «squalenoylation» consists in covalent binding of the squalene with siRNA RET/PTC1 or RET/PTC3 at the 3'-terminus of the sense strand via maleimide-sulfhydryl chemistry. Remarkably, the linkage led to an amphiphilic molecule that self-organized in water as siRNA-SQ RET/PTC (1 or 3) nanoparticles (NPs). These NPs were used for *in vivo* studies. Interestingly, the siRNA RET/PTC (1 or 3)-SQ NPs injected intravenously or intratumoraly in nude mice were able to reduce tumour growth, inhibit oncogene and oncoprotein expressions, induce apoptosis and partially restore differentiation (decrease in Ki67) [15, 19] (Fig. 2). Taken together these results showed that the vectorized siRNAs against the *RET/PTC* junction oncogene could be used in clinical studies to assess their effects in patients with the *RET/PTC* junction oncogene.

Regulation of TTF-1 by Wnt/β-catenin pathway. TTF-1 is a tissue specific transcription factor expressed in epithelial cells of the thyroid and lunga, as well as in certain areas of the brain [20]. TTF-1 is a useful immunohistochemical marker in the diagnosis of thyroid or lung cancers [21–23]. TTF-1 is well detectable in papillary carcinomas and absent in anaplastic carcinomas, therefore it was used as a marker to distinguish between these two types of thyroid neoplasms [23, 24]. The Wnt signaling pathway plays a critical role in the development and organogenesis [25] but this pathway has also emerged as a critical pathway in carcinogenesis [26]. Furthermore, many β-catenin targets have been shown to play an important role in cancer including *c-myc*, cyclin *D1*, matrix metalloproteases, *CD44*, homeodomain containing genes [27].

The Wnt/β-catenin pathway activation is defined by three molecules, a constitutively active serine kinase, called glycogen synthase kinase-3 (GSK-3β), adenomatous polyposis coli (APC) and the scaffolding protein Axin, associated with β-catenin in the cytoplasm. β-catenin is usually phosphorylated by GSK-3β and is subsequently targeted by ubiquitination for rapid proteasome degradation. In the presence of Wnt ligands, Frizzled receptor is activated in the membrane, which leads to the inactivation of GSK-3β through intermediary molecules including the dishevelled and GSK-3β binding protein. This results in the accumulation of β-catenin in the cytoplasm and its subsequent translocation to the nucleus, where it forms a complex with the nuclear transcriptional regulator T-cell factor/lymphoid enhancer factor (TCF/LEF), finally causing the binding of downstream genes and promotion of transcriptional activation.

It has been suggested by many authors that the β-catenin may play a direct role in the dedifferentiation commonly observed in the late-stage disease of PTC [28]. However, the role of canonical Wnt signaling in TTF-1 regulation remains undetermined. Our aim was to investigate whether the Wnt/β-catenin pathway would regulate TTF-1 expression in a papillary thyroid carcinoma model and to examine the mechanism through which this regulation takes place. Using immunocytochemical analysis, RT-qPCR and Western blots studies we found that TTF-1 as well as the major Wnt pathway components were expressed in TPC-1 cells. Knocking-down the Wnt/β-catenin components by siRNAs inhibited both TTF-1 transcript and protein expression. The activation of Wnt signalling by lithium chloride or

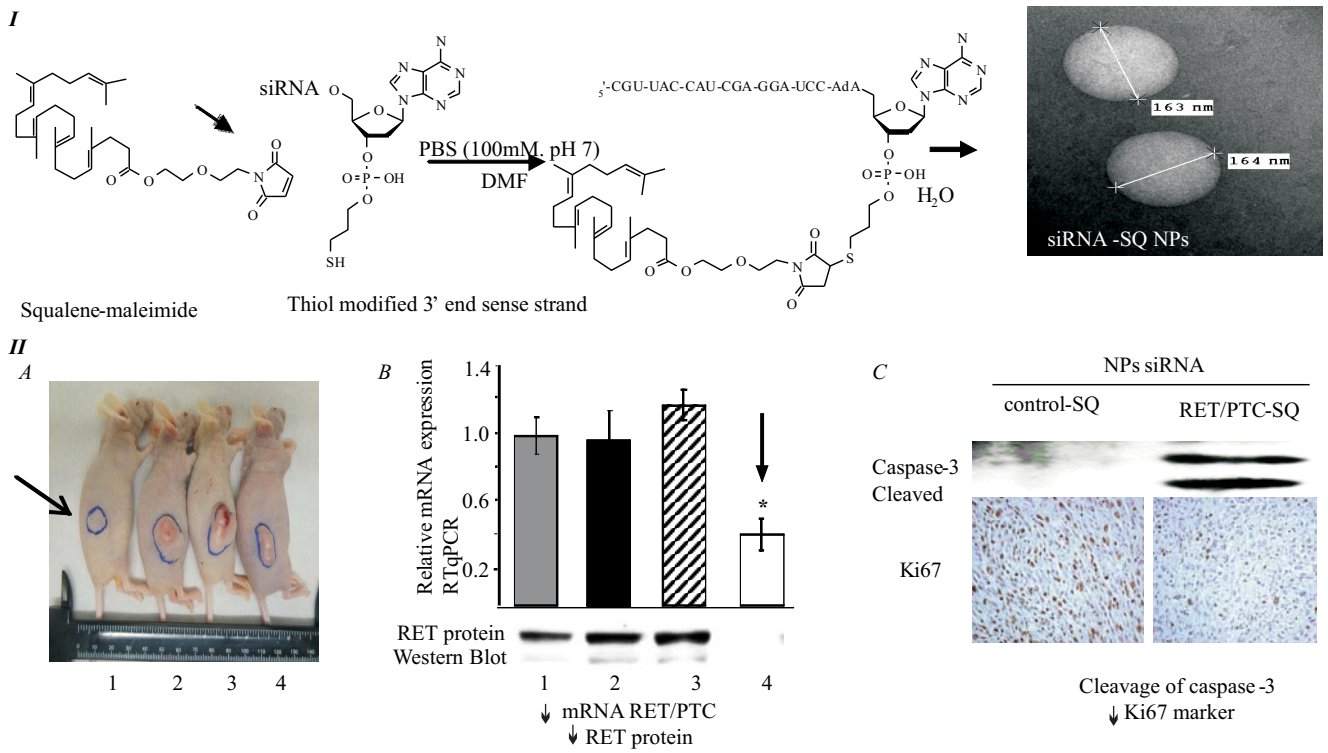

Fig. 2. Summary of the main results obtained *in vivo* after squalenisation of siRNA. *A* – squalenisation of siRNAs (the squalene was covalently coupled to therapeutic siRNAs (siRNA RET/PTC1 or to siRNA RET/PTC3) or to siRNA Control modified in the 3'-end sense strand; the bioconjugation is able to give nanoparticles in water of above 160 nm of diameter); *B – in vivo* studies (when the siRNAs-SQ nanoparticles are injected into nude mice a regression of tumor growth was observed paralleled with a decreases of mRNA RET/PTCs mRNA levels and of RET protein content; moreover, a cleavage of caspase-3 and a decrease of Ki67 dedifferentiation marker were observed in the treated tumors)

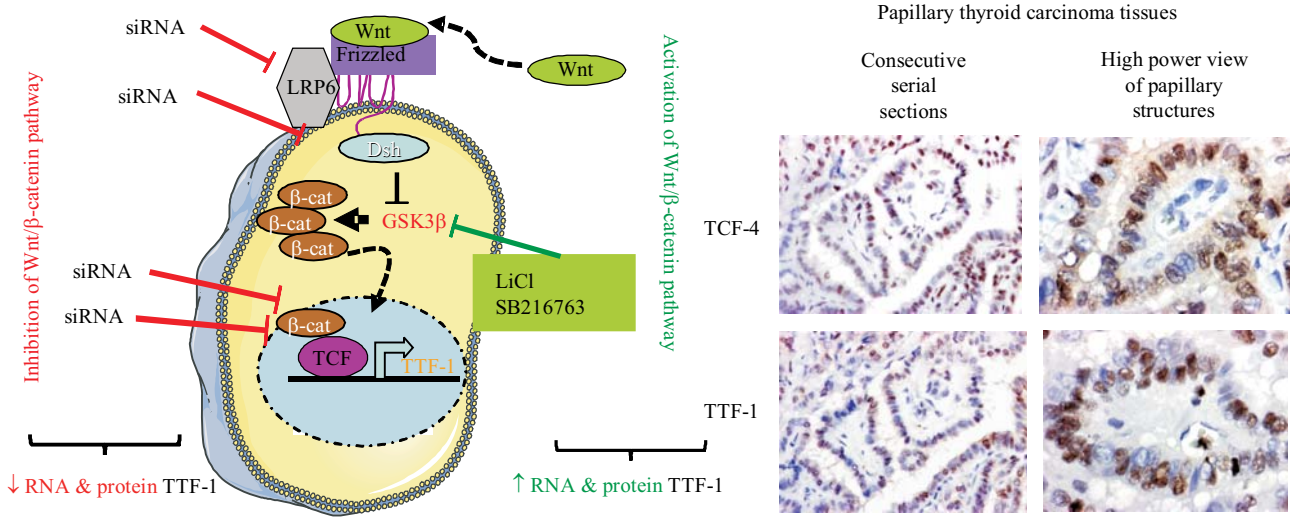

Fig 3. Summary of the main results obtained concerning the regulation of TTF-1 by Wnt/β-catenin pathway: *A* – strategies used to assess the regulation of TTF-1 by Wnt/β-catenin pathway. Two strategies were used; the first one consists in inhibiting the molecular partners of Wnt/β-catenin by siRNA against the major molecular partners LRP6, disheveled (Dsh), β-catenin (β-cat) and TCF. A decreased of mRNA and protein TTF-1 levels were observed. The second strategy is to mimic the activation of these signaling pathways by LiCl or SB216763, both known as inhibitors of GSK-3β activity. An increase of TTF-1 mRNA and protein was observed. By promoter experiments followed by chromatine immunoprecipitation (ChIP) assays an active TCF responsive element was found between 798 and 792 bp upstream the ATG start site. *B*– colocalisation of TCF-4 and TTF-1 transcription factor. Immunochemical studies showed a perfect colocalisation of TCF-4 and TTF-1 in the same area of papillary thyroid carcinoma tissues

SB216763 induced the *TTF-1* gene and protein expression. The functional promoter studies and ChIP analysis showed that the Wnt/β-catenin pathway exerts its effect by means of the binding of β-catenin to TCF/LEF transcription factors at (-798, -792 bp) in TTF-1 promoter. Moreover, immunohistological studies performed on human normal thyroid and PTC tissues revealed that the TTF-1 and transcription factor 4 (TCF4) proteins showed a colocalisation of β-catenin and TCF-4 within the papillary cells [29] (Fig. 3). This study suggests that TTF-1 is a direct transcriptional target of the Wnt/β-catenin signalling pathway and could be investigated in association with a targeted treatment against the *RET/PTC* oncogene in the case of aggressive or persistent PTC.

Conclusions. These studies have a double impact. From a fundamental point of view, this work demonstrates for the first time that Wnt/β-catenin regulates TTF-1 in the papillary thyroid cancer cells through β-catenin-binding to the TCF/LEF-responsive element present in TTF-1 promoter. We suggest that Wnt/β-catenin pathway contributes to the fine-tuning of TTF-1 expression and could have different biological consequences according to the cellular context. We speculate that normal Wnt/β-catenin expression maintains the basal TTF-1 expression and differentiated cell state, whereas the activation of Wnt/β-catenin signaling would have an effect on tumour progression (occurs consequently to mutations within the molecular Wnt signaling partners leading to the continuous activation of β-catenin and thus of TTF-1). From a medical point of view, we hope that the administration of LiCl or other GSK-3β inhibitors or Wnt modulators will stimulate the expression of TTF-1 and, as a consequence, promote the thyroid cells differentiation in pathologies where the TTF-1 expression is lacking or weak. This is of great interest for thyroid or lung cancers where the TTF-1 expression is crucial to maintain cell differentiation.

Concerning the significance of siRNA RET/PTC-SQ NPs, we succeeded in establishing and delivering siRNAs against the *RET/PTC1* and *RET/PTC3* junction oncogenes. This would have clinical application for patients with papillary thyroid carcinoma but further pharmacological and clinical investigations should be done to set-in the remedy of thyroid carcinoma.

We speculate that if we want to offer personalized treatments for cancer patients with junction oncogenes we should counteract dedifferentation due to the *RET/PTC* junction oncogenes by knocking down the oncogenic fusion genes products by siRNA and supplement, if necessary, this targeting therapy by modulating the expression of transcription factor involved in the tumor progression.

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Необходимость воздействия на слитый онкоген RET / PTC3 и Wnt/β-катенин сигнальный путь при лечении папиллярного рака щитовидной железы: результаты восьмилетней работы

Л. Массад-Массад

Резюме

Папиллярный рак щитовидной железы (PTC) является наиболее распространенным злокачественным новообразованием эндокринных желез и часто вызывается облучением. PTC характеризуется парацентрической инверсией в хромосоме 10, приводящей к слиянию с RET нескольких генов, присутствующих в щитовидной железе именно при PTC. Слитые онкогены RET/PTC обнаружены почти в 80 % случаев PTC, наиболее часто встречаются RET/PTC1 и RET/PTC3, при этом их не выявлено в окружающих нормальных тканях, вследствие чего они представляют собой хорошую мишень для стратегий РНК-интерференции. Цель нашей работы состояла, с одной стороны, в ингибировании дедифференцирования с использованием миРНК слитого онкогена RET/PTC и, с другой, – в исследовании роли Wnt/β-катенин сигнального пути в регуляции тканеспецифического фактора транскрипции (TTF-1), важного для дифференциации щитовидной железы. В этой статье кратко представлены основные результаты, полученные в результате восьмилетней работы, указывающие на новую терапевтическую стратегию для папиллярного рака щитовидной железы.

Ключевые слова: карцинома папиллярного щитовидной железы, RET/PTC слитые онкогены, миРНК, опухолевые клетки.

Необхідність впливу на злитий онкоген RET/PTC3 і Wnt/β-катенін сигнальний шлях при лікуванні папілярного раку щитоподібної залози: результати восьмирічної роботи

Л. Массад-Массад

Резюме

Папілярний рак щитоподібної залози (PTC) є найрозповсюдженішим злоякісним новоутворенням ендокринних залоз, який часто спричиняється опроміненням. PTC характеризується парацентричною інверсією в хромосомі 10, яка призводить до злиття з RET декількох генів, що присутні в щитоподібній залозі саме при PTC. Злиті онкогени RET/PTC3 знайдено майже у 80 % випадків PTC, найчастіше зустрічаються RET/PTC31 і RET/PTC3, при цьому їх не виявлено в оточуючих нормальних тканинах, внаслідок чого вони представляють собою гарну мішень для стратегій РНК-інтерференції. Мета нашої роботи полягала, з одного боку, в інгібуванні дедиференціювання з використанням міРНК злитого онкогена RET/PTC та, з іншого, – у дослідженні ролі Wnt/β-катенін сигналь ного шляху в регуляції тканиноспецифічного фактора транскрипції (TTF-1), важливого для диференціювання щитоподібної залози. У цій статті коротко представлено основні результати, от римані в результаті восьмирічної роботи, які вказують на нову терапевтичну стратегію для папілярного раку щитоподібної залози.

Ключові слова: папілярний рак щитоподібної залози, RET/PTC злиті онкогени, міРНК, пухлинні клітини.

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