Medical University of Gdańsk
Faculty of Medicine

Doctoral Thesis

The role of thyroid hormones in the development of colorectal cancer

(pol.) Rola hormonów tarczycy w rozwoju raka jelita grubego.

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Dla Rodziców

Za to, że zrobiliście wszystko i więcej, żebym mogła być tu, gdzie teraz jestem.

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Abbreviations

CRC – colorectal cancer
CSC – cancer stem cells
D 1, 2 and 3 - deiodinases 1, 2 and 3
HCT116 i HT29 – CRC cell lines
PICO – Patients, Interventions, Comparisons, Outcomes
PRISMA – Preferred Reporting Items for Systematic Reviews and Meta-Analyses
SCH - subclinical hypothyroidism
T3 – triiodothyronine
T4 – thyroxine
TH – thyroid hormones
THR – thyroid hormones replacement therapy
THRβ – thyroid hormones receptor β

Keywords

colorectal cancer; cancer risk; colorectal cancer stem cells; hypothyroidism; thyroid hormones; thyroid hormones replacement therapy.

Abstract

Introduction

Colorectal cancer (CRC) is third most frequently diagnosed cancer and second leading cause of cancer-related deaths worldwide(1). Although the treatment of CRC has improved over the last few decades and five-year survival rate for patients with stage I CRC is approximately 92%, it is still one of the most fatal cancers with five-year survival rate drastically dropping to only 13% for patients with stage IV CRC(2).

A widely acknowledged model asserts that most colorectal cancers (CRC) originate from precursor lesions arising from intestinal lining – adenomatous polyps, in a phenomenon referred to as the adenoma-to-carcinoma sequence(3). Following pivotal mutations, an advanced adenoma undergoes a transformation into colorectal cancer. If left undetected, the cancer advances through sequential stages, ranging from stage I to ultimately stage IV.

Several factors have been established as risk contributors for colorectal adenomas and CRC, including obesity, lack of physical activity, smoking, and low-fiber diet (4). On the other hand, certain agents have protective influence on CRC risk. Commonly used medications like aspirin or metformin have already been found to demonstrate preventive effects against CRC carcinogenesis (5–7). Another group of agents known to have interplay with cancer risk and pleiotropic effects are thyroid hormones. However, comprehensive descriptions of the relationship between thyroid hormone imbalance and CRC risk are still lacking in available data.

Thyroxine (T4), the main product of thyroid gland, is an inactive form of thyroid hormones. Deiodinases, specifically type 1 (D1) and 2 (D2), convert T4 into its active form triiodothyronine (T3). Both T3 and T4 can be transformed into inactive metabolites through type 3 deiodinase (D3)(8). Disruption in the function of D3 has been implicated in tumorigenesis of haemangiomas, hepatocellular carcinomas, as well as breast and thyroid cancers. Numerous studies have shown a significant correlation between hypothyroidism and the development of pancreatic, gastric and breast cancer (9–11). The investigation into the association between thyroid gland dysfunction and colorectal cancer has not been that thoroughly explored.

This doctoral thesis consists of two studies – one systematic review and one original study. First of the two entitled "Effects of thyroid hormone imbalance on colorectal cancer carcinogenesis and risk- a systematic review" consolidates all findings and explores the link between thyroid hormone imbalance and risk of colorectal cancer. Second one, entitled "Triiodothyronine lowers the potential of colorectal cancer stem cells *in vitro*" is an original study investigating a novel

function of thyroid hormones signaling in the regulation of cancer stem cells (CSC) of colorectal cancer.

Aims

Publication 1

The aim of this study was to summarize all findings and potentially elucidate the connection between thyroid hormones imbalance and colorectal cancer carcinogenesis and risk.

Publication 2

The aim of this study was to assess the potential impact of triiodothyronine on cancer stem cells of colorectal cancer.

Material and methods

Publication 1

This systematic review was performed according to PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) and PICO (Patients, Interventions, Comparisons, Outcomes) guidelines. On 7th May 2018 the MEDLINE, ClinicalTrials.gov, www.clinicaltrialsregister.eu, and Cochrane Library databases were searched. The following search query was used: "((((thyroid OR hypothyroidism OR hyperthyroidism OR levothyroxine OR hashimoto OR graves OR thyroidectomy)) AND (colon OR colorectal OR CRC)) NOT Hashimoto [Author]) NOT graves [Author]". No filters were active. Two independent researchers screened results. The initial search returned 3054 results. After screening of abstracts, 46 results were chosen for full text analysis, of which 11 met inclusion criteria and were included in the study. Studies were included into analysis if predefined PICO criteria were met. Articles were divided into two categories: cell-line studies and human studies.

Publication 2

This was an original study, in which freshly retrieved CRC tissues from CRC patients were used to isolate and expand CSCs. A total of 27 CRC patients were enrolled into the study in the Department of General, Endocrine and Transplant Surgery. The samples were collected between July 2020 and April 2021. CSCs were also isolated and expanded from two commercially available CRC cell lines, HCT116 and HT29, that were cultured in spherical forms. All subpopulations of CSCs were treated in vitro with T3 hormone and following 3 day-incubation

certain biological properties were determined. For this study data were analyzed in two groups: early (stages I + II) and advanced CRC stage of the patient (stages III + IV).

Statistical analysis was performed using GraphPad Prism. Data were analyzed with non-parametric Mann-Whitney U test or Kruskal-Wallis test followed by Dunn's test as a post hoc procedure and Spearman's rank correlation analysis. $p<0.05$ was considered to indicate a statistically significant difference.

Results

Publication 1

The results of this systematic review can be divided into two groups – results of cell lines studies and human studies. In cell lines studies, the analyzed literature showed that higher level of T3 inhibits colon cancer cells proliferation, promotes differentiation in cultured cells and sharply mitigate tumour formation. What is more, the findings suggest that also the TH receptor B1 plays an important role in CRC progression by regulating PI3K/Akt signaling, which can be a future target in CRC therapy.

Seven analyzed human studies were case-control studies (five) and cohort studies (two). The studies showed that thyroid hormone (TH) replacement therapy is associated with lower risk of CRC (OR 0.59, 95% CI: 0.43-0.82, $p = 0.001$) and after being adjusted for many factors such as age, sex, family history etc. the results remained statistically significant. Moreover, the studies showed that the patients with subclinical hypothyroidism (SCH) with no history of TH replacement therapy had higher risk of CRC compared to patients without any thyroid dysfunction. Furthermore, the SCH was found to be an independent risk factor for colorectal neoplasm.

Publication 2

The results of this study showed that T3 eliminates colorectal CSCs in *in vitro* culture. It caused visible changes in the morphology of spheres derived from both examined CRC cell lines and patients with CRC. The sizes of colonospheres of cells treated with T3 were significantly reduced ($p<0.05$). Furthermore, another assay was carried out to determine the ability of CRC cells to form colonospheres following 72-h-long incubation and then being transferred into fresh medium without T3. It showed that pretreatment with T3 exerted a durable influence on CRC cells. Moreover, the results of the study showed that T3 decreases the viability of colorectal CSCs ($p<0.05$).

Conclusions

Presented studies suggest that thyroid hormones and their imbalance can exert a great impact on the colorectal cancer risk by influencing colon cancer cells proliferation, viability of the cells and their potential to differentiate.

Considering growing use of TH supplementation, it is essential to explore possible connection between the level of supplementation and CRC risk, but also the potential use of thyroid hormones as an adjunct to treat or slow down the progression of colorectal cancer.

Streszczenie (pol.)

Wstęp

Rak jelita grubego (RJG) jest trzecim najczęściej diagnozowanym nowotworem i drugą najczęstszą przyczyną zgonów z powodu nowotworów na świecie(1). Pomimo, że leczenie raka jelita grubego uległo znacznemu postępowi w ciągu ostatnich kilku dekad, a wskaźnik przeżycia pięcioletniego osób z RJG w stadium I wynosi około 92%, jest to nadal jeden z najbardziej śmiertelnych nowotworów, a wskaźnik przeżycia pięcioletniego drastycznie spada (do 13%) u pacjentów z RJG w IV stopniu zaawansowania(2).

Powszechnie uznany model zakłada, że większość nowotworów jelita grubego ma swój początek w zmianach prekursorowych wywodzących się z błony śluzowej jelit – gruczolakach jelita grubego, w zjawisku określanym jako sekwencja gruczolak-rak(3). W wyniku kluczowych mutacji zaawansowany gruczolak ulega transformacji do raka jelita grubego. Jeśli nie zostanie wykryty i usunięty, rak rozwija się w kolejnych stadiach zaawansowania przechodząc kolejno od stadium I do ostatecznie stadium IV.

Ustalono, że czynniki zwiększające ryzyko wystąpienia gruczolaków jelita grubego oraz RJG obejmują otyłość, brak aktywności fizycznej, palenie tytoniu i dietę ubogą w błonnik(4). Z drugiej strony, niektóre czynniki mogą zmniejszać ryzyko wystąpienia nowotworu jelita grubego. Stwierdzono już, że powszechnie stosowane leki, takie jak aspiryna czy metformina, wykazują działanie zapobiegawcze przeciwko RJG(5–7). Kolejną grupą substancji, które odgrywają rolę w ryzku nowotworzenia i mają efekty plejotropwe są horomony tarczycowe. W dalszym ciągu jednak brakuje kompleksowych badań nad związkiem pomiędzy zaburzeniami równowagi hormonalnej tarczycy, a ryzykiem wystąpienia raka jelita grubego.

Tyroksyna (T4), główny hormon produkowany przez tarczycę, jest nieaktywną formą jej hormonów. Dejodynazy, a dokładnie typ 1 (D1) i 2 (D2), przekształcają T4 w jego aktywną formę, trijodotyroninę (T3). Zarówno T3, jak i T4 mogą zostać przekształcone w nieaktywne metabolity poprzez dejodynazę typu 3 (D3)(8). Zaburzenia funkcjonowania D3 powiązano z powstawaniem nowotworów takich jak naczyniaki krwionośne, raki wątrobowokomórkowe, a także raki piersi i tarczycy. Liczne badania wykazały istotną korelację pomiędzy niedoczynnością tarczycy, a rozwojem raka trzustki, żołądka i piersi (9–11). Związek pomiędzy dysfunkcją tarczycy a rakiem jelita grubego nie został dokładnie zbadany.

Niniejsza praca doktorska składa się z dwóch badań. Pierwsze z dwóch zatytułowane „Effects of thyroid hormone imbalance on colorectal cancer carcinogenesis and risk – a systematic review" podsumowuje wszystkie ustalenia i

wyjaśnia związek pomiędzy zaburzeniem równowagi hormonów tarczycy, a ryzykiem rozwoju raka jelita grubego rak. Drugie, zatytułowane „Triiodothyronine lowers the potential of colorectal cancer stem cells *in vitro*" to praca oryginalna opisująca nową funkcję sygnalizacji hormonów tarczycy w regulacji nowotworowych komórek macierzystych (CSC) raka jelita grubego.

Cele

Publikacja 1

Celem tego badania było podsumowanie wszystkich dostępnych publikacji i potencjalne wyjaśnienie związku pomiędzy zaburzeniami równowagi hormonów tarczycy a karcynogenezą i ryzykiem raka jelita grubego.

Publikacja 2

Celem pracy była ocena potencjalnego wpływu trójjodotyroniny na nowotworowe komórki macierzyste raka jelita grubego.

Materiał i metody

Publikacja 1

Niniejszy przegląd systematyczny przeprowadzono zgodnie z wytycznymi PRISMA (*Preferred Reporting Items for Systematic Reviews and Meta-Analyses) i PICO (Patients, Interventions, Comparisons, Outcomes*). W dniu 7 maja 2018 r. przeszukano następujące bazy danych: MEDLINE, ClinicalTrials.gov, www.clinicaltrialsregister.eu i Cochrane Library. Zastosowano następujące zapytanie: „((((tarczyca LUB niedoczynność tarczycy LUB nadczynność tarczycy LUB lewotyroksyna LUB Hashimoto LUB Graves LUB tyroidektomia)) ORAZ (okrężnica LUB jelito grube LUB CRC)) NIE Hashimoto[Autor]) NIE Graves [Autor]" . Żadne filtry nie były aktywne. Wyniki oceniło dwóch niezależnych badaczy. Początkowe wyszukiwanie dało 3054 wyniki. Po selekcji abstraktów do analizy pełnotekstowej wybrano 46 badań, z czego 11 spełniło kryteria włączenia i zostało wykorzystane do dalszych analiz. Badania włączono do analizy, jeśli spełnione zostały wcześniej zdefiniowane kryteria PICO. Artykuły podzielono na dwie części: badania na liniach komórkowych i badania na ludziach.

Publikacja 2

W badaniu tym do izolacji i namnażania CSC wykorzystano komórki świeżo wyizolowane z tkanek raka jelita grubego pobranych od pacjentów z RJG. Do badania włączono 27 pacjentów Kliniki Chirurgii Ogólnej, Endokrynologicznej i Transplantacyjnej. Próbki pobrano w okresie od lipca 2020 r. do kwietnia 2021 r. Wyizolowano i namnożono także CSC z dwóch dostępnych na rynku linii

komórkowych RJG:CRCRJG:, HCT116 i HT29, które hodowano w postaci sfer. Wszystkie subpopulacje CSC traktowano in vitro hormonem tarczycy T3 i po 3-dniowej inkubacji określono ich właściwości biologiczne. Na potrzeby badania dane analizowano w dwóch grupach: wczesnego (stadia I + II) i zaawansowanego raka jelita grubego (stadia III + IV).

Analizę statystyczną przeprowadzono przy użyciu GraphPad Prism. Dane poddano nieparametrycznemu testowi U Manna-Whitneya lub testowi Kruskala-Wallisa, a następnie testowi Dunna jako procedurze post hoc i analizie korelacji rang Spearmana. Uznano, że $p<0,05$ wskazuje statystycznie istotną różnicę.

Wyniki

Publikacja 1

Wyniki tego przeglądu systematycznego należy podzielić na dwie grupy – wyniki badań na liniach komórkowych i badań na ludziach. W badaniach linii komórkowych, wykazano, że wyższy poziom T3 hamuje proliferację komórek raka jelita grubego, sprzyja różnicowaniu hodowanych komórek i znacznie spowalnia proces powstawania nowotworu. Co więcej, badania sugerują, że również receptor dla hormonów tarczyc B1 odgrywa ważną rolę w progresji RJG poprzez regulację sygnalizacji PI3K/Akt, co może być przyszłym celem w leczeniu RJG.

Siedem analizowanych badań na ludziach to badania kliniczno-kontrolne (pięć) i badania kohortowe (dwa). Badania wykazały, że terapia zastępcza TH wiąże się z niższym ryzykiem RJG (OR 0,59, 95% CI: 0,43-0,82, $p = 0,001$), a po uwzględnieniu wielu czynników, takich jak wiek, płeć, wywiad rodzinny itp., wyniki były istotne statystycznie. Ponadto badania wykazały, że u pacjentów z subkliniczną niedoczynnością tarczycy (SCH), którzy nie stosowali w przeszłości terapii hormonalnej, ryzyko wystąpienia RJG było wyższe w porównaniu z pacjentami bez dysfunkcji tarczycy. Ponadto stwierdzono, że SCH jest niezależnym czynnikiem ryzyka nowotworu jelita grubego.

Publikacja 2

Wyniki tego badania wykazały, że trijodotyronina (T3) eliminuje komórki macierzyste raka jelita grubego w hodowli in vitro. Spowodowała ona (T3) widoczne zmiany w morfologii sfer pochodzących zarówno z badanych linii komórkowych RJG jak i komórek wyizolowanych od pacjentów z RJG. Rozmiary kolonosfer komórek traktowanych T3 uległy znacznemu zmniejszeniu ($p<0,05$). Ponadto przeprowadzono badania w celu określenia zdolności komórek RJG do tworzenia kolonosfer po 72-godzinnej inkubacji z T3, a następnie przeniesieniu do świeżej pożywki bez T3. Wykazano, że wstępne podanie T3 wywiera przetrwały wpływ na komórki RJG. Co więcej, wyniki badania wykazały, że T3 zmniejsza żywotność CSC jelita grubego ($p<0,05$).

Wnioski

Z przedstawionych badań wynika, że hormony tarczycy oraz zaburzenia ich równowagi mogą wywierać wpływ na ryzyko raka jelita grubego poprzez wpływ na proliferację komórek raka, ich żywotność oraz potencjał do różnicowania.

W świetle rosnącego stosowania suplementacji TH istotne jest zbadanie możliwego związku pomiędzy poziomem suplementacji a ryzykiem RJG oraz możliwość potencjalnego wykorzystania hormonów tarczycy w leczeniu lub spowolnieniu progresji raka jelita grubego.

Introduction

Colorectal cancer (CRC) is third most frequently diagnosed cancer and second leading cause of cancer-related deaths worldwide(1). Although the treatment of CRC has improved over the last few decades and the five-year survival rate for people with stage I CRC is approximately 92%, it is still one of the most fatal cancers with five-year survival rate drastically dropping to 13% for patients with stage IV CRC(2).

A widely acknowledged model asserts that most colorectal cancers (CRC) originate from precursor lesions arising from intestinal lining – adenomatous polyps, in a phenomenon referred to as the adenoma-to-carcinoma sequence(3). Following pivotal mutations, an advanced adenoma undergoes a transformation into colorectal cancer. If left undetected, the cancer advances through sequential stages, ranging from stage I to ultimately stage IV.

Recent studies suggest that cancer transformation depends not only on genetic changes in cells or changes within the tumor microniche, but largely on overall hormonal homeostasis, in which T3 and T4 hormones play key roles. It has been shown that thyroid hormone homeostasis influences the pathogenesis and progression of many types of cancers, including colorectal cancer (CRC)(9,10,12). If untreated, both hyperthyroidism and hypothyroidism may correlate with susceptibility to the development of cancer(13–16).

The active form of thyroid hormones- triiodothyronine (T3) is a transcription factor that regulates the expression of genes for proteins responsible for the control of metabolic processes, differentiation, proliferation, and apoptosis in various types of tissues. The detailed role of thyroid hormones in the carcinogenesis of colorectal cancer has not been described. Although the literature describes the positive effects of long-term hormonal therapy with a synthetic T4 analogue(17), no detailed analysis of the cellular proteins involved in the sensitivity of cancer cells to the given treatment has been performed. Little is known about the influence of thyroid hormones on the properties of cancer stem cells (CSC), which have been shown to play a role in the pathogenesis and progression of CRC.

This doctoral thesis consists of two studies – one systematic review and one original study. First of the two entitled "Effects of thyroid hormone imbalance on colorectal cancer carcinogenesis and risk- a systematic review" consolidate all findings and investigates the link between thyroid hormone imbalance and risk of colorectal cancer. Second one, entitled "Triiodothyronine lowers the potential of colorectal cancer stem cells *in vitro*" is an original study exploring a novel function of thyroid hormones signaling in the regulation of cancer stem cells (CSC) of colorectal cancer.

Aims of the studies

Publication 1

Effects of thyroid hormone imbalance on colorectal cancer carcinogenesis and risk – a systematic review.

The aim of this study was to summarize all findings and potentially elucidate the connection between thyroid hormones' imbalance and colorectal cancer carcinogenesis and risk.

Publication 2

Triiodothyronine lowers the potential of colorectal cancer stem cells in vitro. The aim of this study was to assess the potential impact of triiodothyronine on cancer stem cells of colorectal cancer.

Results

Publication 1

The results of this systematic review can be divided into two groups- results of cell lines studies and human studies. In cell lines studies, the analyzed literature showed that higher level of T3 inhibits colon cancer cells proliferation, promotes differentiation in cultured cells and sharply mitigate tumour formation. The studies also demonstrated that CRC-CSCs are highly sensitive to intracellular T3, while D3 maintains CRC stem cells in the undifferentiated status and enhances tumour growth. What is also interesting a higher level of D3 was found in adenomatous epithelium and CRC. The studies also analyzed the correlation between TRB1 protein expression and clinicopathological characteristics of the CRC cases. It resulted in statistically significant ($p=0.045$) inverse correlation between the tumour size and TRB1 expression. The findings could suggest that the TH receptor B1 plays an important role in CRC progression, and potentially can be a future target in CRC therapy. The only study that showed the results opposite to the presented above was conducted by Lee et al. and showed that T4 enhanced cell proliferation in both CRC cell lines used (HCT 116 and HT-29). However, even the author pointed out that T3 and T4 may present a different effect and may have opposite effects on colorectal cancer cells in terms of their development and proliferation.

Seven analyzed human studies were case-control studies (five) and cohort studies (two). The studies showed that TH replacement therapy is associated with lower risk of CRC (OR 0.59, 95% CI: 0.43-0.82, p = 0.001). Consistent with the widely acknowledged prevalence of hypothyroidism being higher in women than in men, the research indicated a more frequent use of levothyroxine supplementation among female control subjects compared to their male counterparts (8.2% vs. 2%, respectively, p<0.0001). Although the decrease in colorectal cancer (CRC) incidence among men with levothyroxine supplementation did not reach statistical significance (OR= 0.75, 95% CI: 0.42-1.36, p = 0.35), a statistically significant association was observed in women (OR= 0.54, 95% CI: 0.38-0.75, p < 0.0001). However, as a response to the first study, Friedman et al. conducted a study, which showed that rectal cancer risk was lower in men supplementing levothyroxine (OR= 0.66, 95% CI: 0.45-0.97, p = 0.03). The research also examined the correlation between the timing of thyroid hormone (TH) replacement initiation and colorectal cancer (CRC) risk. The findings revealed that the protective effect becomes more pronounced with the duration of therapy. Moreover, the studies showed that the patients with subclinical hypothyroidism (SCH) with no history of TH replacement therapy had higher risk of CRC compared to patients without any thyroid dysfunction. What is also interesting is the fact that patients with SCH were more likely to have advanced colonic lesions and CRC compared to euthyroid subjects (p = 0.028 and p = 0.036, respectively). Furthermore, the SCH was found to be an independent risk factor for colorectal neoplasm.

Publication 2

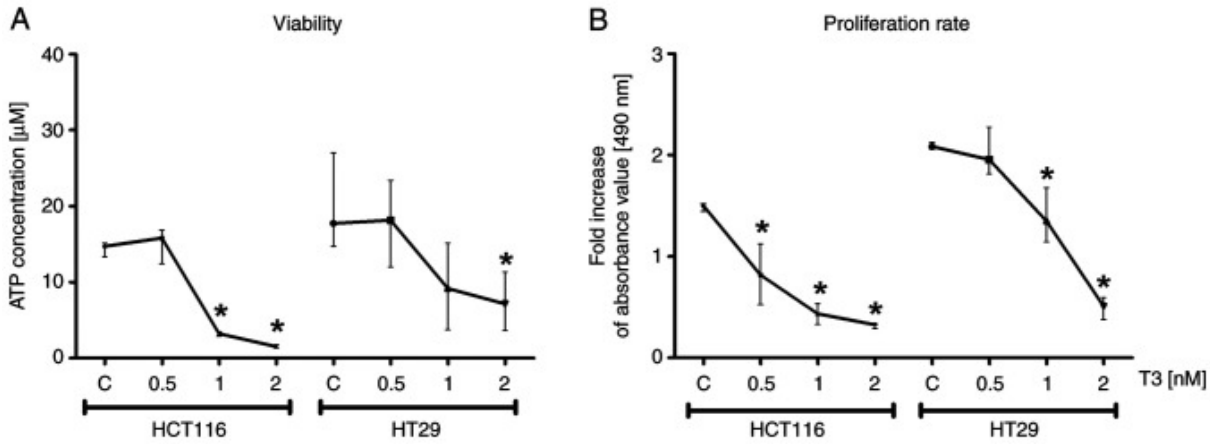
Cancer stem cells (CSCs) play a key role in the development and progression of colorectal cancer. The results of this study consist of analysis of phenotype, cell cycle, survival and proliferation abilities of CSCs in a 3D model of CSCs cultures treated with T3.

Triiodotyronine caused visible changes in the morphology of spheres derived from both examined CRC cell lines (HCT116 and HT29) and cells of the patients with CRC. What is more, the study showed that the effect T3 caused is permanent. After one week of incubation in fresh medium without T3, CRC cells should form colonospheres with the typical morphology and size, but it was found that the T3 pre-treated colonospheres were evidently smaller (p<0.05) in comparison to primary spheres.

It was also revealed that the number of 7AAD-positive cells among cultured cells increased after incubation with T3 concentration-dependent manner

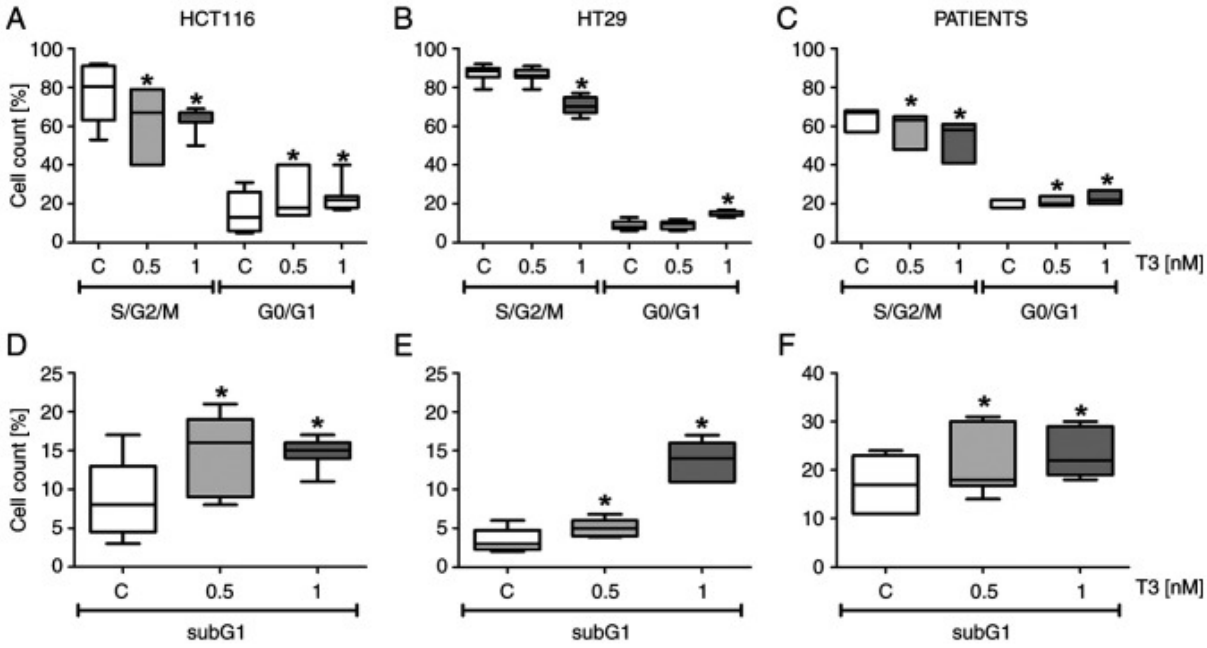
(p<0.05). 7-AAD dye is used to evaluate the viability of the cells as it is excluded by living cells but binds selectively to the DNA of damaged permeabilized cells.

The viability of CRC cells of HCT116 and HT29 cell lines was also analyzed by the assessment of total levels of cellular ATP. The results confirmed that the increased concentration of T3 in culture reduced viability of colonospheres (p<0.05). Moreover, it was revealed that also the proliferation rate was significantly decreased following T3 treatment in comparison to control cells (p<0.05).



Evaluation of (A) viability and (B) proliferation abilities of colorectal cancer cells. Colonospheres formed from HCT116 and HT29 cell lines were treated with thyroid T3 hormone (0.5, 1 and 2 nM) and analyzed. Statistical significance was showed with Kruskal-Wallis test or Mann-Whitney U test. Bars represent median and interquartile range. *P<0.05 vs. untreated control samples (C)

The results also illustrated connection between T3-treatment and the cell cycle. G0/G1 cell cycle growth arrest was observed after the incubation of spheres with T3, while the number of cells in S and G2-M phases (active phases of the cycle) was markedly reduced in comparison to untreated controls.



Distribution of cells in cell cycle phases. Cells of colonospheres formed from (A and D) HCT116 and (B and E) HT29 cell lines and (C and F) cancer cells isolated from human CRC tissue treated with thyroid T3 hormone (0.5 or 1 nM) or untreated control cells (C) were analyzed. (A-C) Y-axis presents cell frequency (%) in active (S/G2/M) or in G0/G1 phases or (D-F) dying cells in subG1 phase. Statistical significance was showed with Kruskal-Wallis test or Mann-Whitney U test. Data presented as box and whiskers represent the median with min-max values. *P<0.05 vs. untreated control cells (C), n=6 for each experimental group.

Conclusions

Presented studies suggest that thyroid hormones and their imbalance can exert an impact on the colorectal cancer risk by influencing colon cancer cells proliferation, viability of the cells and their potential to differentiate.

The first study was designed to summarize all findings and potentially elucidate the connection between thyroid hormones imbalance and colorectal cancer carcinogenesis and risk. Despite promising results of the first report (Rennert et al. 2010) describing the protective effects of TH replacement therapy on CRC risk, only 11 papers analyzing this subject were available. After analyzing all of these it was found that both cell lines studies and human studies results suggest that there is a connection between TH imbalance and CRC risk. Those conclusions could be an essential foundation for potential animal studies and

more human studies, which could determine whether TH axis could become one of the targets in molecular therapy.

The second study was designed as the outcome of the systematic review and its results. The aim was to assess the impact of T3 on CRC cells. Considering the results, it could be hypothesized that thyroid hormones exert an impact on the fate of colorectal cancer stem cells. CSCs are considered to be responsible for tumor initiation, development, maintenance, metastasis, and recurrence. Their sensitivity to T3 could be considered as a potential therapeutic target for designing anticancer drugs. Considering that T3 was found to target CSCs it can be also deliberated that T3 supplementation for CRC patients may influence the content of tumour microenvironment. The adjunctive therapy with thyroid hormones might help to improve the efficacy of conventional chemotherapy, however further research is needed.

Considering growing use of TH supplements, it is essential to explore possible connection between level of supplementation and CRC risk and the potential use of thyroid hormones to treat or slow the progression of colorectal cancer.

List of publication and bibliometrics

Publication 1
Effects of thyroid hormone imbalance on colorectal cancer carcinogenesis and risk – a systematic review
Rostkowska O, Spychalski P, Dobrzycka M, Wilczyński M, Łachiński AJ, Obołończyk Ł, Sworczak K, Kobiela J.
Endokrynol Pol. 2019;70(2):190-197.
doi: 10.5603/EP.a2019.0007.
Bibliometrics: Impact Factor: 1.322 MNiSW: 70

Publication 2
Triiodothyronine lowers the potential of colorectal cancer stem cells in vitro
Rostkowska O, Olejniczak-Kęder A, Spychalski P, Szaryńska M, Kobiela J.
Oncol Rep. 2023 Jan;49(1):21.
doi: 10.3892/or.2022.8458.
Bibliometrics: Impact Factor: 4.2 MNiSW: 70

Summary of blibliometrics:
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Effects of thyroid hormone imbalance on colorectal cancer carcinogenesis and risk — a systematic review

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Abstract

Colorectal cancer (CRC) is the second leading cause of cancer-related death. The prevalence of colorectal neoplasm is increasing. Many studies have shown that thyroid dysfunction may be connected with the higher risk of pancreatic and breast cancer, but only a few described the role of thyroid dysfunction and thyroid hormone (TH) replacement in the development and risk of CRC. The aim of this study is to summarise all findings and potentially elucidate the connection between TH imbalance and colorectal cancer.

The systematic review was conducted according to PICO and PRISMA guidelines. We searched MEDLINE, ClinicalTrials.gov, www.clinicaltrialsregister.eu, and Cochrane Library databases using the following keywords: "((((thyroid OR hypothyroidism OR hyperthyroidism OR levothyroxine OR hashimoto OR graves OR thyroidectomy)) AND (colon OR colorectal OR CRC)) NOT hashimoto[Author]) NOT graves[Author])". No filters were used.

Of total of 3054 articles identified by the search strategy, 11 met PICO criteria and were included into the review. Four of those were on cell lines and seven were human studies. Analysis of the included studies revealed an elevated risk of CRC in patients with hypothyroidism with aORs ranging from 1.16 (95% CI: 1.08–1.24, $p < 0.001$) to 1.69 (95% CI: 1.21–2.36, $p = 0.002$). Moreover, TH replacement therapy has a protective effect for CRC risk with aOR ranging from 0.60 (95% CI: 0.44–0.81, $p = 0.001$) to 0.92 (95% CI: 0.86–0.98, $p = 0.009$). THs seem to play a role in colorectal carcinogenesis. Further studies are warranted to define the exact role of thyroid hormone imbalance in prevention and treatment of CRC. **(Endokrynol Pol 2019; 70 (2): 190–197)**

Key words: *colorectal cancer; cancer risk; hypothyroidism; thyroid hormones; levothyroxine*

REVIEW

Introduction

According to the American Cancer Society colorectal cancer (CRC) is the third most common cancer diagnosed in both men and women (excluding skin cancers) and the third leading cause of cancer-related deaths worldwide [1]. Although the treatment of CRC has improved over the last few decades, it is still expected to cause about 8% of cancer-related deaths in 2018 [1]. The five-year relative survival rates for people with stage I CRC is approximately 92% [2]. However, it decreases to 12% for patients in stage IV of the disease [2]. Therefore, it is still crucial to explore the nature of CRC development, progression, and risk factors.

The majority of CRCs develop from adenomatous polyps arising from the intestinal lining. This phenomenon is called adenoma-carcinoma sequence [3]. Numerous variables have been confirmed as risk factors of colorectal adenomas and CRC, such as obesity, physical inactivity, smoking, and low-fibre diet [4]. On the other hand, there are multiple agents showing a protective influence on risk of CRC. Widely used drugs like aspirin or metformin have already been found to have a preventive action against tumourigenesis of CRC [5, 6]. However, data on TH imbalance and risk of CRC are still not comprehensively described.

Thyroxine, the main product of thyroid gland (T4), is an inactive form of TH. Type 1 (D1) and 2 deiodinases (D2) convert it into T3, which is its active form. Both T3 and T4 can be transformed into inactive metabolites via type 3 deiodinase (D3) [7]. Deregulation of the D3 function has been implicated in tumorigenesis of haemangiomas, hepatocellular carcinomas, and breast and thyroid cancer [8–10].

Multiple studies have shown a significant connection between hypothyroidism and pancreatic, gastric, and breast cancer [11, 12]. The association between the dysfunction of thyroid gland and CRC was not investigated as fully. Therefore, the aim of this study is to perform a systematic review on the association between THs imbalance and CRC risk and treatment.

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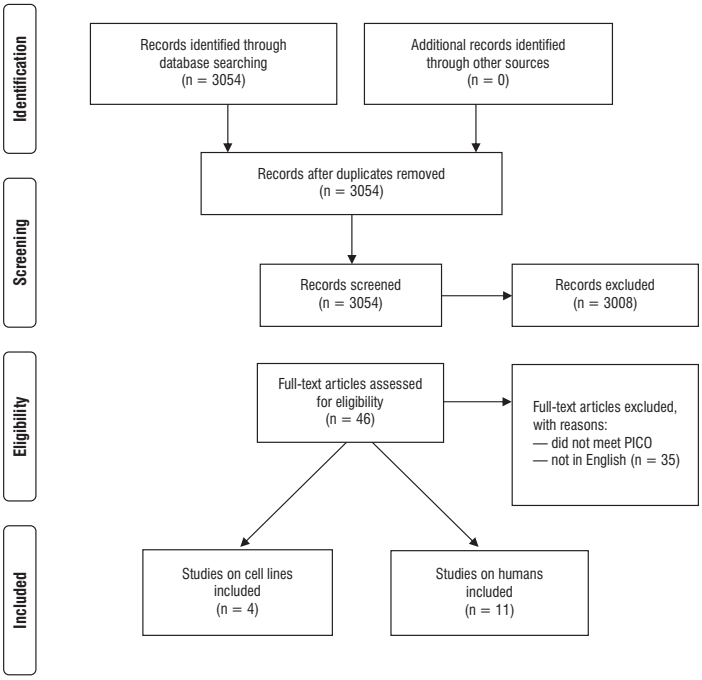

Figure 1. *PRISMA protocol for data acquisition*

A systematic review

Search strategy and study acquisition

This systematic review was performed according to PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) and PICO (Patients, Interventions, Comparisons, Outcomes) guidelines. On 7th May 2018 the MEDLINE, ClinicalTrials.gov, www.clinicaltrialsregister.eu, and Cochrane Library databases were searched. The following search query was used: "((((thyroid OR hypothyroidism OR hyperthyroidism OR levothyroxine OR hashimoto OR graves OR thyroidectomy)) AND (colon OR colorectal OR CRC)) NOT hashimoto[Author]) NOT graves[Author]". No filters were active. Two independent researchers screened results. The initial search returned 3054 results. After screening of abstracts, 46 results were chosen for full text analysis, of which 11 met inclusion criteria and were included in the study. A flowchart of study inclusion is presented in Figure 1.

Inclusion and exclusion criteria

Studies were included into analysis if predefined PICO criteria were met (Tab. I). Articles were dichotomised

Table I. *PICO criteria used in the study*

Part A	
PICO	Description
Patients	Cell studies: Studies on colorectal cancer cell lines And/or studies on colorectal cancer stem cells
Intervention	Use of: T3, T4, or D3 inhibitors
Comparisons	Cell lines exposed to change in thyroid hormone levels were compared to cell lines not exposed to such changes
Outcomes	Rates of differentiation and proliferation
Part B	
PICO	Description
Patients	Patients with diagnosed colorectal cancer Patients with diagnosed thyroid diseases (subclinical hypothyroidism, hyperthyroidism)
Intervention	–
Comparisons	Patients exposed to higher or lower levels or thyroid hormones were compared to patients exposed to normal levels of thyroid hormones
Outcomes	Colorectal cancer risk

Table II. *Cell lines studies*

First author Year of the study	Type of studied cells	The aim of the study	Mechanism	Outcomes	Conclusions
Dentice M. et al. 2012 [21]	Colon cancer cell lines	The role of D3 in the regulation of T3 signalling in cancer cells	Absence of D3 increases level of T3	Higher level of T3 inhibits colon cancer cells proliferation and promotes differentiation in cultured cells	Regulation of T3 may be targeted to reduce the oncogenic effects of β-catenin in intestinal cells
Catalano V. et al. 2015 [13]	Colorectal cancer stem cells	Influence of D3 and T3 on colorectal cancer stem cells	Depletion of D3 increases intracellular thyroid hormone concentration	Higher level of T3 induces cell differentiation and sharply mitigate tumour formation	Combined action on intracellular T3 and chemotherapy could improve colorectal cancer treatment
Lee Y.-S. et al. 2018 [22]	Colorectal cancer cells	Influence of T4 on colorectal cancer cells	T4 induces nuclear B-catenin accumulation, as well as high cyclin D1 and c-Myc	T4 promotes B-catenin activation and cell-proliferation in colorectal cancer	An applicable therapeutic strategy including T4 regulation could be considered
Zhu L. et al. 2016 [17]	Normal colon mucosa cells, human colorectal cancer cells	The role of TRB1 in the CRC tissues and cells	TRB1 works via the PI3K/Akt pathway	TRB1 plays a critical role in the progression of CRC	TRB1 could be a potential target for CRC therapeutics

CRC — colorectal cancer

into two categories: cell-line studies (Tab. I, part A) and human studies (Tab. I, part B).

Evidence extraction and synthesis

Information from studies that met eligibility criteria on cell lines was abstracted by two independent researchers into Table II. Information from studies that met eligibility criteria on humans was abstracted by two independent researchers into Table III.

Results

A total of 11 studies met predefined PICO criteria and were dichotomised into two categories: cell-line studies and human studies. No studies on animals were available.

Cell lines studies

A total of four studies were included into the review. Results are summarised in Table II. Included studies were based on varying methodology. One study was on CRC stem cells, and three studies were on CRC cells. Characteristics of included studies are summarised in Table II.

A study by Catalano et al. focused on the role of THs in CRC stem cell differentiation and their sensitivity to chemotherapy [13]. CRC stem cells (CR-CSC) are a small group of cells with self-renewing potential and the ability to induce tumour growth [14, 15]. CSCs are highly insensitive to chemotherapy and other kinds of treatment, which can lead to inefficient management of the disease [16]. One of the factors determining the potential survival of stem cells, the possibility of their self-renewal and resistance to chemotherapy, is the Wnt signalling pathway. It controls the sequence of events leading to the neoplastic transformation of colon mucosa. Catalano et al. proved that Wnt-b-catenin pathway drives an inverse, coordinated regulation of D2 and D3 in CRC cells. Moreover, they demonstrated that CR-CSCs are highly sensitive to intracellular T3, while D3 maintains CRC stem cells in the undifferentiated status and enhances tumour growth. T3 appears effective in inducing cell differentiation, affects the Wnt target-related genes, and makes the CR-CSCs more sensitive to chemotherapy. The results of the study suggest that the effect of chemotherapy on T3-induced cells could be superior to the effect chemotherapy on normal CRC cells.

Zhu et al. investigated the role of TH receptor β1 (TRβ1) in the progression of CRC [17]. Mutations of TRs have already been found to play a role in many kinds of cancers (such as breast, thyroid, hepatocellular, and renal clear cell cancer) [9, 10, 18, 19]. Additionally, mice in which mutated TR gene have been introduced develop follicular thyroid cancer with enhanced capabilities of metastasising to the lungs [20]. Zhu et al. analysed 222 tissue samples from patients with CRC. Decreased expression of TRβ1 mRNA in colon and rectum adenocarcinoma samples was found in 89.7% of samples. Furthermore, the study analysed the correlation between TRβ1 protein expression and clinicopathological characteristics of the CRC cases. It

Table III. *Human studies*

Author Year published Years of study	Study design	Number of patients	Subclinical hypothyroidism (SCH)	Hypothyroidism	Hyperthyroidism	Thyroid replacement therapy (THR)
Colorectal cancer risk						
Guifang M et al. 2014 2008–2012	Case-control	CRC:non-CRC 1:3 273:819	aOR 1.689 (95% CI: 1.207–2.362) p = 0.002			
Boursi B et al. 2015 1995–2013	Case-control	CRC:non-CRC 1:4 20990:82054	aOR 1.16 (95% CI: 1.08–1.24) p < 0.001		aOR 1.21 (95% CI: 1.08–1.36) p = 0.001	aOR 0.92 (95% CI: 0.86–0.98), p = 0.009 **THR 5–10 years** aOR 0.88 (95% CI: 0.79–0.99), p = 0.03 **THR over 10 years** aOR 0.68 (95% CI: 0.55–0.83), p < 0.001
Rennert G et al. 2010	Case-control; survey	CRC:non-CRC 2566:2566 Respone rate 70%:59%				aOR 0.60 (95% CI: 0.44–0.81), p = 0.001
Friedman G et al. 2011 1994–2008	Case-control	CRC:non-CRC 1:50 Colon 12207:608296 Rectum 4729:235925				**Men:** Colon: aOR 0.87 (95% CI: 0.71–1.07), p = 0.18 Rectum: aOR 0.66 (95% CI: 0.45–0.97), p = 0.03 **Women:** Colon: aOR 0.90 (95% CI: 0.81–1.01), p = 0.06 Rectum: aOR (95% CI: 0.78)

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Table III. *Human studies*

Author Year published Years of study	Study design	Number of patients	Subclinical hypothyroidism (SCH)	Hypothyroidism	Hyperthyroidism	Thyroid replacement therapy (THR)
Cancer risk (CRC was a subgroup)						
Chan Y et al. 2016	Prospective cohort study	3836	aSHR3 1.19 (95% CI: 1.00–1.42) p = 0.048			
Chen YK et al. 2013 1998–2010	Case-control	HT1-nonHT 1:4 1521:6084		aHR 4.76 (95% CI: 1.36–16.6)		
Chen YK 2013	Longitudinal cohort study	GD2-nonGD noncancer 1:4 5025:20100			aHR 0.61 (95% CI: 0.30–1.24)	

[1]Hashimoto thyroiditis; [2]Graves-Basedov disease; [3]Adjusted sub-hazard ratio; CI — cinfidence interval; CRC — colorectal cancer; OR — odds ratio; HR — hazard ratio

resulted in a statistically significant (p = 0.045) inverse relation between the tumour size and TRβ1 expression, i.e. the expression was lower in tumours larger than 5 cm. Moreover, the results showed that overexpression of TRβ1 inhibits CRC cell proliferation and suppresses the migration of the cells by inhibiting PI3K/Akt signalling. All the findings could suggest that TRβ1 plays an important role in CRC progression by regulating PI3K/AKT pathway and can be a future target in CRC therapy.

Another cell line study was conducted by Dentice et al. and investigated the role of D3 in the regulation of T3 signalling in cancer cells [21]. The study explored the correlation between D3 expression in CRC cells and B-catenin complex. The results showed that B-catenin up-regulation of D3 leads to reduced levels of T3, which inhibits differentiation and promotes cellular proliferation. A higher level of D3 was also found in adenomatous epithelium and CRC. The conclusion of the study was that affecting B-catenin-D3-T3 pathway could be a potential target for new CRC therapies.

Only Lee et al. presented results in contrast to those described above [22]. Their study investigated the role of T4 in CRC cell lines and its effect on B-catenin activation. According to the study, T4 enhanced cell proliferation in both CRC cell lines used (HCT 116 and HT-29) by hyper-activation of Wnt/B-catenin cascade. Higher level of B-catenin was connected with faster progression of the disease and unfavourable survival. However, Lee et al. pointed out that T4 and T3 may present different functions in the progression of CRC, and the relationships between THs require deeper exploration.

Human studies

A total of seven studies on humans were included into the analysis. Of those, four analysed only CRC risk and three analysed CRC as a subgroup. Five of the seven were case-control studies. The remaining two were cohort studies. Only one study was prospective. The characteristics of the included studies are summarised in Table III.

Rennert et al. in a case-control study on 2566 matched pairs showed that TH replacement therapy is associated with lower risk of CRC, with OR = 0.59 (95% CI: 0.43–0.82, p = 0.001) [23]. After being adjusted for age, sex, use of aspirin and statins, sports activity, family history of CRC, ethnic group, and level of vegetable consumption, the results remained statistically significant with aOR = 0.60 (95% CI: 0.44–0.081, p = 0.001). In accordance with the well-known fact that hypothyroidism is much more common in women than in men, the study showed that levothyroxine supplementation was more frequent in female than in male control subjects (8.2% *vs.* 2.0%, respectively, p < 0.0001). While reduction of CRC in men with

levothyroxine supplementation was not statistically significant (OR = 0.75, 95% CI: 0.42–1.36, p = 0.35), the association in women was (OR = 0.54, 95% CI: 0.38–0.75, p < 0.0001). After subgroup analysis, the study showed reduced risk of CRC in postmenopausal women supplementing levothyroxine (OR = 0.53, 95% CI: 0.37–0.74, p < 0.001). Furthermore, analysis was adjusted for hormone (oestrogen) replacement therapy (HRT), and the results showed a statistically significant effect among non-HRT users (OR = 0.49, 95% CI: 0.33–0.74, p < 0.001) but not among HRT users (OR = 0.90, 95% CI: 0.35–2.36, $P_{interaction}$ = 0.19). In the fully adjusted model for postmenopausal women, levothyroxine supplementation was related with significant reduction in CRC risk (OR = 0.60, 95% CI: 0.4–0.81, p = 0.001).

As a response to the study by Rennert et al., Friedman et al. published data on 12,207 patients with colon cancer and 4729 with rectum/rectosigmoid cancer [24]. Each patient was matched with 50 controls. According to this study, rectal cancer risk was lower in men supplementing levothyroxine for at least five years (OR = 0.66, 95% CI: 0.45–0.97, p = 0.03). Risk reduction of colon cancer was not statistically significant. The risks for postmenopausal women, both HRT users and non-users, were slightly reduced but not statistically significant.

Boursi et al. published data from a nested case-control study on a large UK population, showing similar results [25]. A total of 20,990 CRC patients were matched with 82,054 controls. The study focused on determining the risk of developing CRC as a result of thyroid dysfunction with TH replacement, as well as without it. Patients with TH replacement history were included into the study. Analysis of CRC risk was stratified by time of THs supplementation (0–6 months, 6–12 months, 1–5 years, 5–10 years, and > 10 years before index data) because the process of CRC tumorigenesis is multistep and lasts from 10 to 15 years. Adjusted OR for CRC in thyroxine-users was 0.88 (95% CI: 0.79–0.99, p = 0.03) and 0.68 (95% CI: 0.55–0.83, p<0.001) for treatment initiated 5 to 10 years and more than 10 years before index data, respectively. The results were adjusted for age, male sex, diabetes mellitus, smoking, alcohol use, and NSAID use. The analysis of the association between timing of TH replacement initiation and CRC risk showed that the protective influence is increased with cumulative duration of therapy. Moreover, the patients with clinical or subclinical hypothyroidism with no history of THR had higher risk of CRC compared to patients without any thyroid dysfunction (OR = 1.16, 95% CI: 1.08–1.24, p < 0.001). Hyperthyroidism was also associated with an increased CRC risk (OR = 1.21, 95% CI: 1.08–1.36, p = 0.001).

A case-control study by Guifang et al. analysed 273 colorectal neoplasm patients matched with 819 controls [26]. The prevalence of subclinical hypothyroidism (SCH) was significantly higher in group of patients with colorectal neoplasm, compared to those without (p < 0.01). Colorectal neoplasms were found in 67 (34.9%) subjects in the SCH group, which was more than that in euthyroid group (206, 24.1%, p = 0.002). Moreover, patients with SCH were more likely to have advanced colonic lesions and CRC compared with euthyroid subjects (p = 0.028 and p = 0.036, respectively). After adjusting for blood pressure, body mass index, history of hypertension, and smoking, an association still existed between colorectal neoplasm and SCH (OR = 1.689, 95% CI: 1.21–2.36, p = 0.002). The authors concluded that a strong association between SCH and colorectal neoplasm was identified. SCH was found to be an independent risk factor for colorectal neoplasm.

Furthermore, three studies on cancer risk and TH imbalance contained data on CRC. A prospective cohort study by Chen et al. on 3863 patients reported adjusted hazard ratio (HR) of 1.19 (95% CI: 1.00–1.42, p = 0.048) of CRC for patients with subclinical hypothyroidism (SCH) [27]. Chen et al. in 2013 published data from a case-control study on 1521 patients with Hashimoto disease (HD) matched with 6084 non-Hashimoto and reported adjusted HR of 4.76 of developing CRC (95% CI: 1.36–16.6. p < 0.05) [28]. The same authors reported data from a longitudinal cohort study on 5025 patients with Graves-Basedow disease (GD) matched with non-GD patients, which revealed a protective effect of this disease on CRC risk with aHR of 0.61 (95% CI: 0.30–1.24, p < 0.001) [29]

Discussion

The development of preventive strategies in CRC is a subject of extensive research. The relationship between CRC risk and some widely used medications such as metformin or statins has been described and showed promising results [5, 6, 30]. Another possible factor influencing the development of CRC is an imbalance of thyroid hormones resulting from diseases such as: subclinical hypothyroidism, Hashimoto's disease, and Graves-Basedow disease, and their treatment. The first report on the protective effects of THs replacement therapy on CRC risk was published by Rennert et al. in 2010 [23]. Further studies were in accordance with those results, showing that high concentrations (but within range) of THs diminishes the risk of CRC. Despite those promising initial results, to date only 11 papers analysing this subject are available, both on molecular and epidemiological level. Therefore, more thorough examination of this subject seems warranted.

Cell lines studies

In vitro studies analysed in our review showed encouraging results; however, to date only four papers were published. Despite being based on varying methodologies, two of four came to similar conclusions, suggesting that higher concentrations of T3 achieved by inhibition of D3 serve as an inhibitor of tumourigenesis. Moreover, one study explored the role of TRb1 in cancer progression, which could become a target for molecular therapy. Only one study by Lee showed unfavourable results, i.e. that T4 promotes cancer proliferation. However, T4 is not a biologically active form, and therefore these results should be interpreted cautiously. All included cell-line studies bear limitations of a very specific design, which analyses only certain scenarios, such as inhibition of D3. It would be of great interest to compare the influence of T3 versus T4 on proliferation and differentiation of CRC cell lines and CRC CSC, to fully understand the role of TSH-T4-T3 axis on CRC development. Additional points of interest are the influence of TSH on peripheral cells as well as rT3, which contributes to 0.9% of THs in the bloodstream [31]. Furthermore, the presented studies analyse the influence of inhibition of only one enzyme: D3 deiodinase, while there are more enzymes that take part in metabolic pathways of T4 and T3, such as deiodinase type 1 and 2. Inhibition or activation of those enzymes could potentially influence the tumourigenesis of CRC and therefore could be a future target for molecular therapy. Some existing studies describe in detail the relationships and interdependences between THs and CRC, but straightforward conclusions on the effects of those relationships are lacking. Those conclusions (i.e. whether T3 or T4 promote or inhibit carcinogenesis) could be an essential foundation for potential animal and human studies. In summary, initial *in vitro* studies on the influence of THs on CRC show promising results. However, further research is needed — both to confirm available results and to explore other possible associations between the THs axis and CRC. This will enable clear conclusions to be drawn, which would be the rationale for animal and human studies.

Animals studies

The search strategy of this review identified no studies conducted on animals, despite the fact that similar scenarios were tested with metformin, aspirin, or statins [6, 32]. Animal studies investigating the effect of metformin showed that it reduces aberrant crypt foci formation and downregulates tumour angiogenesis. Aspirin has been shown to prevent colorectal cancer and CRC metastasis in mice. Such studies could contribute to the state of knowledge of association of levothyroxine supplementation and CRC risk.

Human studies

Studies on humans included in this analysis show consistent and promising results. Concentrations of THs within the upper limit of normal correlate with lower risk of CRC in all of the analysed studies. However, the quality of available data is moderate due to retrospective study designs, which are susceptible to multiple biases. Moreover, there are numerous factors contributing to cancer risk in general and CRC risk specifically, such as age, sex, diet, region of residence, or medications. On the other hand, epidemiology of thyroid diseases is also age- and sex-dependent. Therefore, it is essential to perform adjusted analyses of CRC risk that include both typical risk factors (such as age and sex) and less typical such as common medications that are known to influence CRC risk, including metformin and aspirin. The included studies report adjusted ORs; however, factors included into adjustment are varied. Moreover, there is a trend towards supplementation of T4 both in healthy individuals and in individuals with SCH [33]. Therefore, in light of growing use of TH supplements, it is essential to explore possible connections between level of supplementation and CRC risk. Furthermore, existing studies did not analyse dose escalation of T4 supplementation and did not report information on how the dose was established. This is of great interest because dose escalation of other drugs that serve a protective role in CRC risk enhanced this effect. Another interesting aspect of TH replacement is the time of exposure. Typically, patients who suffer from hypothyroidism are diagnosed in their twenties, while the average age of CRC diagnosis is 60+ years old. Therefore, time of exposure is substantially longer in comparison to other drugs that may serve a protective role in CRC, such as the aforementioned metformin or aspirin.

The main strength of this study is its novelty. This is, to our knowledge, the first available systematic review on the influence of TH imbalance on CRC. It systematically summarises findings on the topic in a Cochrane-style manner, which is another important quality.

The main limiting factor of the present study is the volume of included studies. This is due to scarce amounts of series available in the literature. This limits the possibilities to draw clear and definitive conclusions, a trend is visible nonetheless. Moreover, some of the studies acquired data via questionnaires in a retrospective manner and therefore are susceptible to recall bias. Most of the studies included into the review were case-control studies and therefore were susceptible to time window bias, i.e. a bias resulting from measuring exposure over unequal time intervals.

In conclusion, based on our literature review, there is growing evidence suggesting a possible role of euthyreosis in CRC prevention and treatment. Further studies are required to validate these results.

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Triiodothyronine lowers the potential of colorectal cancer stem cells *in vitro*

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Abstract. Cancer stem cells (CSCs) play a key role in the development and progression of colorectal cancer (CRC), but the influence of triiodothyronine (T3) on the biological regulation of CSCs remains unclear. In the present study, it was reported that T3 exerts significant impact on CSCs of two CRC cell lines cultured in the form of colonospheres. It was observed that the incubation of colonospheres with T3 decreased the viability, proliferative and spherogenic potential of cancer cells ($P<0.05$). In addition, increased apoptotic rate of CRC cells treated with T3 was revealed. Furthermore, T3-treated colonospheres were more likely to move into silenced pool in G0/G1 phase of the cell cycle. The smaller sizes of colonospheres observed after the treatment with T3 confirmed this conclusion. T3 could lower the proportion of primitive cells which supply the pool of proliferating cells within spheres. Thyroid receptors THRα1 and THRβ1 and two deiodinases (DIO2 and DIO3) were affected by T3 in manner depended on clinical stage of cancer and CRC cell line used for analysis. In summary, the present study uncovered a novel function of thyroid hormones signaling in the regulation of the CSCs of CRC, and these findings may be useful for developing novel therapies by targeting thyroid hormone functions in CRC cells.

Introduction

Colorectal cancer (CRC) is the second leading cause of cancer-related death worldwide with 916,000 deaths in 2020 according to the WHO and studies of CRC markedly attract attention in both basic and clinical sciences. The discovery of novel therapeutic targets in cancer cells and increase of anticancer drug choices have significantly improved CRC treatment strategies in recent years. Several studies demonstrated that hormonal context of the patient plays a crucial role in carcinogenesis, progression and treatment outcome of breast, lung, colon, liver and prostate cancers (1-4). It was revealed that hyperthyroidism can increase the risk of cancer transformation and promote tumor cells proliferation and migration. Concurrently, hypothyroidism was found to exert opposite effects and may induce apoptosis of cancer cells (5,6).

Despite the fact that cancer stem cells (CSCs) major functions include driving the incidence, mortality and metastasis of cancers (7), the relationship between thyroid gland function, thyroid hormones activity and CSCs properties remains controversial and requires further studies. Since the existence of CSCs was demonstrated for most of cancer types, including CRC (8), the analysis of relations between thyroid hormones and CSCs transformation appears to be crucial in order to improve success rate of therapeutic strategies and survival rates of CRC patients.

The aim of the present study was to investigate the impact of triiodo-L-thyronine (T3) on the features of CRC CSCs since these aspects of CRC microenvironment are still not fully understood. Analysis of phenotype, cell cycle, survival and proliferation abilities of CSCs was conducted in a 3D model of CSCs cultures. Colonospheres are considered to represent a widely used reliable surrogate assay to assess CSCs ability as it has been previously reported by the authors (9,10). The evaluation of the influence of cancer microenvironment elements on cancer cells, their proliferative and metastatic capabilities is a challenge to be addressed.

Materials and methods

Study design. Freshly isolated CRC tissues from CRC patients were used to isolate and expand CSCs. CSCs were also isolated and expanded from two commercially available CRC cell lines, HCT116 and HT29, that were cultured in spherical forms. All subpopulations of CSCs were treated *in vitro* with T3 thyroid hormone and following 3 day-incubation certain biological properties were determined. All results were compared with untreated control cells. All human CRC fragments involved into the current study were

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obtained from surgically resected specimens from CRC patients. Written informed consent for receiving samples from the specimens was obtained from the patients. Ethical approval (approval no. (NKBBN/203/2020) was obtained from The Independent Bioethics Committee of Medical University of Gdansk (Gdansk, Poland). The development of a second neoplastic disease and previous chemo- and/or radiotherapy were exclusion criteria in the present study. All included patients underwent cancer staging and treatment according to EURECCA guidelines (11). Additionally, cancers were staged according to the tumor, node, metastases (TNM) classification (8th edition) (12).

For the purpose of this study data were analyzed in two groups: early (stages I + II) and advanced CRC stage (stages III + IV). The rationale for this stratification is based on differing prognosis and treatment guidelines between early (predominantly surgery) and advanced cancers (surgery with adjuvant chemotherapy or neoadjuvant chemoradiation in rectum) (11).

CRC primary cell lines isolation and expansion. A total of 27 CRC patients were enrolled into the study in the Department of General, Endocrine and Transplant Surgery, Medical University of Gdansk, Poland. The samples were collected between July 2020 and April 2021. The solid tumor fragments were placed into sterile PBS supplemented with Antibiotic-antimycotic solution and immediately processed in culture. All chemical supplements and compounds were purchased from Sigma-Aldrich; Merck KGaA. The growth factors were purchased from R&D Systems, Inc. CRC fragments were washed in serum-free Dulbecco's modified Eagle medium (DMEM)-F12 with antibiotic-antimycotic agent. Afterwards, all specimens were sectioned into smaller pieces (1-2 mm^3) to improve impregnation with collagenase (20 ng/ml) and hyaluronidase (20 ng/ml) for 90 min. Next, cells were filtrated through a 70-μm cell strainer. Primary colon spheroid cultures (SC) were obtained in serum-free stem cell medium (SCM): DMEM-F12 medium supplemented with ITS Liquid Media Complement (1X), BSA (4 mg/ml), glucose (3 ml/ml), Hepes (5 mM), L-glutamine (2 nM), progesterone (20 nM), putrescine (9.6 μg/ml), Heparin (4 μg/ml), EGF (20 ng/ml), bFGF (20 ng/ml), and Antibiotic-antimycotic solution (1X). The composition of SCM was previously established by our group (9,10). Only early passaged SCs were used for analysis in the current study.

HCT116 and HT29 cell lines expansion. Two human CRC cell lines (HT29 and HCT116; (obtained from American Type culture Collection) were used. Cells were tested for *Mycoplasma* contamination. The adherent form of these cell lines was expanded routinely in McCoy's medium supplemented with 10% FBS, 1% penicillin-streptomycin and 2 mM L-glutamine. The cells were passaged with the use of trypsin 2-3 times/week when they achieved 80% confluency. For the need of the current study, CRC cell lines were cultured as colonospheres (spherical 3D forms, tumorospheres) in SCM of the same composition as medium used for CSCs from CRC patients. HCT116 and HT29 cell lines needed at least three passages to obtain the appropriate spherical forms to be included into further analyses.

Cell treatment. HCT116 and HT29 cells ($8x10^5$/ml) and cancer cells isolated from CRC patients were seeded in 24-well ultra-low attachment plates and maintained in SCM. Spheres were treated for 3 days with thyroid hormone T3 (3,3',5-triiodo-L-thyronine; Sigma-Aldrich; Merck KGaA) and incubated at 37˚C under a humidified atmosphere of 5% CO_2. To prepare T3 solutions, 1.0 ml of 1.0 N NaOH to 1 mg of T3 (powder form) was added, gently swirled to dissolve the powder and 49 ml of sterile medium was then added, according to the manufacturer's instructions. All T3 solutions were prepared shortly before use.

Due to limited number of cells (particularly obtained from CRC patients) certain experiments were conducted with only chosen T3 concentrations. The cells maintained in the SCM without T3 were used as control cells. Initially, T3 concentrations for the present study were established according to literature data (0.5, 1 or 2 nM) (13-16). Our first analyses indicated that T3 in concentrations higher than 1 nM causes cell death, thus if a selection had to be made lower T3 concentrations (0.5 or 1 nM) were used. Specifically, the T3 concentrations used are clearly presented on each Fig. and in figure legends.

The analysis of CRC cells' phenotype with flow cytometry. CRC cell lines and cancer cells isolated from CRC patients were stained with monoclonal antibodies (BD Biosciences) characteristic for some CSC-specific antigens: anti-CD29-APC (clone MAR4, IgG1κ; cat. no 559883), anti-CD44-FITC (clone C26, IgG2bκ; cat. no 555478). Additionally, anti-CD133/2-PE (clone 293C3, IgG2bκ; cat. no 130-113-186) monoclonal antibody (Miltenyi Biotec, Inc.) was used. Cells were incubated for 30 min in the dark at room temperature (RT), washed and resuspended with PBS containing 1 mM EDTA for FACS analyses which were performed using FACS Calibur flow cytometer (BD Biosciences) and BD CellQuest Pro 6.0 software. The frequency of positive cells was compared with untreated control cells. To set a threshold of positive signal, unstained cells were used. FACS data are presented as a percentage count of cells with particular phenotype within population set on FSC/SSC plot with excluded small and dead cells.

Cell death assay (7AAD). Following the T3 treatment of HCT116 and HT29 cells and cancer cells isolated from CRC patients the proportion of dead cells in our samples was evaluated. For this purpose, 7AAD Via Probe (BD Biosciences) was used. The cells were incubated for 30 min at RT with 10 μm of a dye, washed with PBS and prepared for FACS analysis. To set a threshold of positive signal, unstained cells were used. The frequency of $7AAD^-$ positive cells was compared with untreated control cells.

Cell cycle analysis. After T3 treatment, $5\text{-}10x10^6$ HCT116 and HT29 cells and cancer cells isolated from CRC patients were washed twice in PBS (Sigma-Aldrich; Merck KGaA), fixed with 70% ethanol on ice and stored at -20˚C until analysis (up to 7 days). Next, cells were treated with ribonuclease to remove any contaminating RNA molecules. Afterwards, the DNA of cells was stained with propidium iodide (50 μg/ml) (PI; Sigma-Aldrich; Merck KGaA). The

fluorescence of the PI-stained cells was analyzed by flow cytometry (FACSCalibur™; BD Biosciences) and the internal control was untreated cells. To set a threshold of positive signal, unstained cells were used.

Proliferation assay. HCT116 and HT29 cells ($1.5x10^4$/ml were seeded in 96-well low attachment plates in SCM, then newly formed spheroids were treated with T3 (0.5, 1 or 2 nM) in 3 replicates for each option. The whole experiment was repeated twice. Non-treated cells were negative control. After 3 days, cell proliferation was assessed by colorimetric Cell Tilter 96® Aqueous One Solution Cell Proliferation Assay (Promega Corporation), according to the manufacturer's instructions. Briefly, 20 μl of Cell Titer 96® Aqueous One Solution Reagent were added into each well of the 96-well assay plate containing the samples in 200 μl of culture medium. After 4 h of incubation at 36˚C the absorbance at 490 nm was recorded using a microplate reader (BioTek Instruments, Inc.).

Viability assay. HCT116 and HT29 cells ($1.5x10^4$/ml) were seeded in 96-well low attachment plates in SCM, then colonospheres were treated with T3 (0.5, 1 or 2 nM) in 3 replicates for each option. The whole experiment was repeated twice. Non-treated cells were used as negative control. After 3 days total levels of cellular ATP were assessed to analyze the cell viability with the use of Luminescent ATP Detection Assay kit (Abcam). The luminescent ATP assay protocol involves lysis of the cell sample, addition of luciferase enzyme and luciferin, and measurement of the emitted light using a microplate-based luminometer (BioTek Instruments, Inc.). This kit irreversibly inactivates ATPases during the lysis step, ensuring that the luminescent signal obtained truly corresponds to the endogenous levels of ATP. The procedure was conducted according to the manufacturer's instructions. Briefly, detergent solution was added to each well and incubated for 5 min at RT to lyse and stabilize ATP. Subsequently, substrate solution was added and after 10 min incubation in dark at RT the luminescence signal was detected. The concentration of ATP in each sample was estimated according to the standard curves acquired after analysis of standard dilutions. Finally, results were presented in μM units.

Colonosphere formation and quantification. The colonospheres derived from HCT116 and HT29 cells or cells isolated from CRC patients were cultured in sphere-forming media and treated with T3 for 3 days. Then the diameter of at least 50 spheres of each experimental group was measured with an inverted light microscope (CKX53) coupled with a digital camera Olympus SC50 (Olympus Corporation). Untreated cells were used as internal control.

Secondary sphere formation ability. After 3 days of treatment with T3, cells ($5x10^4$/ml) were pooled, suspended and seeded in fresh SCM in 96-well low attachment plates. Spheres derived from HCT116 and HT29 cells or cells isolated from CRC patients were monitored by measuring the maximal outgrowth of sphere's diameter after 1 week. Untreated cells were used as internal control. Images were captured using an inverted microscope (CKX53) coupled with digital camera Olympus SC50 (Olympus Corporation).

Western blot analysis. Cell lysates were prepared from colonospheres obtained from both treated and control HCT116 and HT29 cells and cancer cells isolated from CRC patients. After treatment, cells were incubated for 30 min on ice in a RIPA lysis buffer (Sigma-Aldrich; Merck KGaA) supplemented with protease and phosphatase inhibitor cocktail; then centrifuged at 16,000 x g for 10 min at 4˚C. Protein concentration in the lysates was measured with Bradford reagent (Sigma-Aldrich; Merck KGaA). Protein samples (10 μg) were loaded to 4-20% Mini-PROTEAN® TGX™ Precast Protein Gels (Bio-Rad Laboratories, Inc.) and electroblotted onto a PVDF membrane with the use of the Trans-Blot Turbo system (Bio-Rad Laboratories, Inc.). Membranes were incubated with 5% non-fat milk in 1% TBST buffer for 1 h at RT. Afterwards, the membranes were incubated overnight at 4˚C with the following primary antibodies (purchased from Thermo Fisher Scientific, Inc.): rabbit polyclonal anti-THRα1 antibody (1:500; cat. no BS-6221R), rabbit polyclonal anti-THRβ1 antibody (1:100; cat. no PA1213A), rabbit polyclonal anti-DIO2 antibody (1:1,000; cat. no PA549631) and rabbit polyclonal anti-DIO3 antibody (1:1,000; cat. no PA526537). On the next day, blots were washed with TBST and incubated for 1.5 h at RT with HRP-conjugated anti-rabbit IgG antibody (1:10,000; cat. no 1706515; Bio-Rad Laboratories, Inc.). Anti-GAPDH peroxidase-conjugated IgM antibody (1:50,000; cat. no G9295; Sigma-Aldrich; Merck KGaA; 1 h at RT) was used as the loading control. The membranes were washed and subsequently subjected to luminol reagents (Bio-Rad Laboratories, Inc.). The chemiluminescence signal was measured with ImageQuant LAS 500 (Cytiva). Changes in protein level were assessed by densitometric scanning of the bands (ImageQuant™ TL 10.1 analysis software (Cytiva) and corrected for GAPDH loading control. Each experiment was performed in triplicate. Proteins with molecular weights 51, 43, 60, 34 and 37 kDa for THRα1, THRβ1, DIO2 in dimeric form, DIO3 and GAPDH, respectively, were analyzed.

Statistical analysis. Statistical analysis was performed using GraphPad Prism (version 6.05; GraphPad Software, Inc.). Data were subjected to non-parametric Mann-Whitney U test or Kruskal-Wallis test followed by Dunn's test as a post hoc procedure and Spearman's rank correlation analysis. $P<0.05$ was considered to indicate a statistically significant difference. Data in figures are presented as the median ± interquartile range or median with min-max values.

Results

In the present study, early stage CRC consisted of 2 (7.5%) stage I cancer patients and 11 (40.5%) stage II cancer patients. Group of advanced stage consisted of 12 (44.5%) stage III cancer patients and 2 (7.5%) stage IV cancer patients. In our study population, cancer was localized in cecum (11%), ascending colon (15%), transverse colon (7.5%), descending colon (7.5%), sigmoid (33%), rectum (22%) and one of the patients (4%) had two CRC in two different segments of large bowel. In detail 14 patients had lymph node metastases (stage III or IV) and 2 patients had distant metastases at the time of diagnosis (stage IV).

Table I. Clinicopathological characteristics of CRC patients included into the present study.

Cancer stage	I-II	III-IV
Total number	13	14
Sex		
Male	7	8
Female	6	6
Age, years	73.4	71.4
Mean (min-max)	(56-89)	(59-96)
Body mass index	27.5	25
Mean (min-max)	(21-30)	(20-30)
$CD133^+$ (%)[a]		
Mean (min-max)	31 (7-89)	28 (7-91)

[a]Samples whose CSCs survived in culture were included into analysis.

Patient-derived spherical cultures contained variable proportion of CSCs bearing CD133 marker (ranging from 7 to 91%), thus the samples were divided according to CD133 median value (which was 20%) into 2 groups: with high (43±19%) and low (12±4.4%) $CD133^+$ cells count. Apparently, the $CD133^+$ cells count was significantly different between these groups ($P<0.0001$).

T3 eliminates colorectal CSCs in vitro culture. Fresh CRC specimens were collected from 27 patients and it was possible to establish spherical cultures from the cancer tissue of 21 (77.8%) patients, in the remaining cultures cancer cells did not survive *ex vivo* due to bacterial contamination. All functional tests were conducted with cells obtained after the first passage since it has been already revealed that the phenotype of CSCs along the expansion was stable (9). The additional clinicopathological information concerning patients included into the present study are presented in Table I.

Both HCT116 and HT29 CRC cell lines and cancer cells from patients were cultured in a form of colonospheres which are highly enriched with CSCs, as previously reported by the authors (10,17). All cell populations were subjected to cytometric analysis of phenotype following the treatment with T3 thyroid hormone. The proportion of CSCs with commonly used CSC-like markers, namely CD133, CD44 and CD29, was evaluated. The number of $CD133^+$ cells in culture of spheroids obtained from all cancer cells populations did not change significantly (Figs. 1 and 2 and S1), with two exceptions (Fig. 1A).

The general count of $CD133^+$ CSCs in colonospheres representing tissue of patients with CRC of stages I and II did not differ significantly from colonospheres derived from tissues of advanced cancer patients (stages III and IV), thus these groups were divided according to the number of $CD133^+$ median value (into Low and High groups). The statistically significant difference in the percentage of $CD133^+$ expressing cells in colonospheres derived from patients representing both Low and High groups was confirmed ($P<0.05$; Fig. 1). The presented division of patients' samples (Fig. 1) demonstrated that both groups with early and advanced cancer contained samples with high and low count of these primitive cells. This information is of interest since CSCs are known to be responsible for the most serious events of carcinogenesis. Additionally, the analysis of correlation revealed that the amount of untreated and treated $CD133^+$ cells did not depend directly on clinical stage of CRC ($R=0.249$; $P=0.25$) (Fig. S2). Furthermore, all spherical populations were analyzed with respect to the presence of CD29 and CD44 integrins. It was observed that CSCs in colonospheres were more likely to change the percentage of cells bearing another analyzed marker in response to T3 treatment. It was noted that CRC colonospheres treated with T3 had increased count of $CD133^+CD44^-CD29^+$ cells ($P<0.05$). The alterations in count of $CD133^+CD44^-CD29^+$ cancer cells were a mirror image (Fig. 1C) compared with $CD44^+$ counterparts (Figs. 1B and C and 2C and D).

T3 in all concentrations caused visible changes in the morphology of spheres derived from both examined CRC cell lines and cells of patients with CRC. The sizes of colonospheres were significantly reduced when the cells were treated (Fig. 3) with T3 ($P<0.05$) and the number of dead cells and debris in the medium increased following the treatment.

Additionally, another assay was carried out to determine the ability of CRC cells to form colonospheres following 72-h-long incubation of T3-treated cells. Following T3 treatment cells were transferred into fresh medium without T3 (Fig. 4). After 7 days, the effects of T3 on the 'secondary sphere' formation ability were examined, reflecting the tumor-initiating capacity and susceptibility of the CSC-like cells to T3. Both CRC cell lines and cells of patients with CRC presented similar results. Pretreatment with T3 exerted the constant influence on CRC cells. After one week of incubation in fresh medium without T3, CRC cells should form colonospheres with the typical morphology and size, but it was found that the T3 pre-treated colonospheres were evidently smaller ($P<0.05$) in comparison to primary spheres. Additionally, numerous freely floating cells or small aggregates were observed.

T3 decreases the viability of colorectal CSCs. The percentage of non-viable cells following T3 treatment was evaluated by flow cytometric assay using 7-AAD dye (Figs. 5 and S3), which is excluded by living cells, but binds selectively to the DNA of damaged permeabilized cells. It was revealed that the number of 7AAD-positive cells among cultured cells increased after incubation with T3 in concentration-dependent manner ($P<0.05$). Similar results were observed for colonospheres derived from both CRC cell lines and cells isolated from patients with CRC. These results were confirmed by the analysis of cell cycle using PI (Figs. 6D-F and S4). The increased proportion of cells in subG1 phase was noted after the incubation of cells with T3 in different concentrations in comparison to untreated control. At both concentrations of T3 the fraction of cells undergoing apoptosis (in subG1 phase) increased significantly up to 22±4.53% (median ± SD) for 1 nM T3-treated cells of patients (Fig. 6).

The distribution of cells in various phases of the cell cycle was determined in colonospheres' cultures of HCT116 and HT29 cell lines and cancer cells obtained from CRC patients. Generally, the proportion of cells in active phases (S/G2/M) was higher in comparison to cells representing quiescence

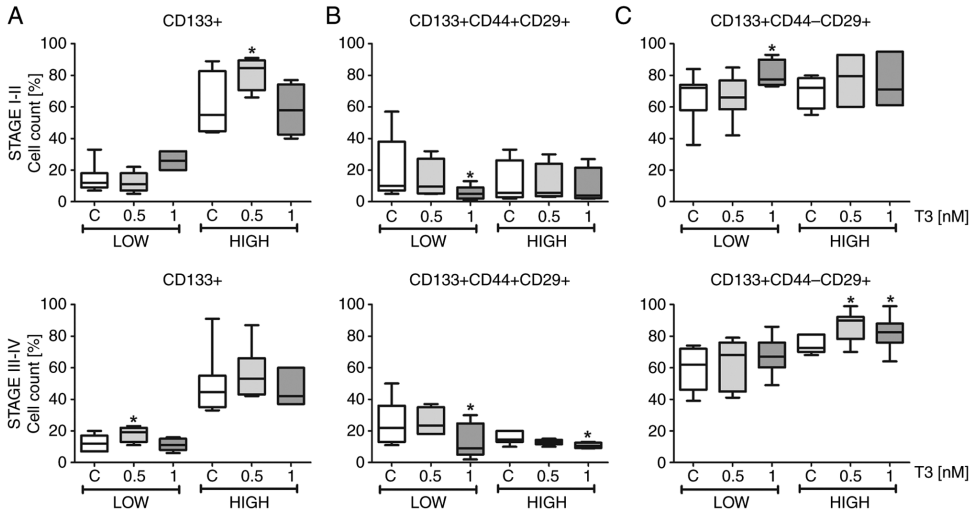

Figure 1. Phenotype of cancer cells isolated from human CRC tissue. The cytometric analysis of human CRC cells treated with thyroid T3 hormone (0.5 or 1 nM). Samples of patients were divided into groups according to the proportion of $CD133^+$ cells (with Low-er or High-er than median value of general population) and in relation to cancer staging (I-II vs. III-IV). The frequency of cells of given phenotype (A. $CD133^+$ or B. $CD133^+CD44^+CD29^+$ or C. $CD133^+CD44^-CD29^+$) is presented on Y-axis (%) and compared with untreated control cells. Statistical significance was showed with Kruskal-Wallis test or Mann-Whitney U test. Bars and whiskers represent the median with min-max values. *P<0.05 vs. untreated control cells (C), n=6-12 for each experimental group. CRC, colorectal cancer.

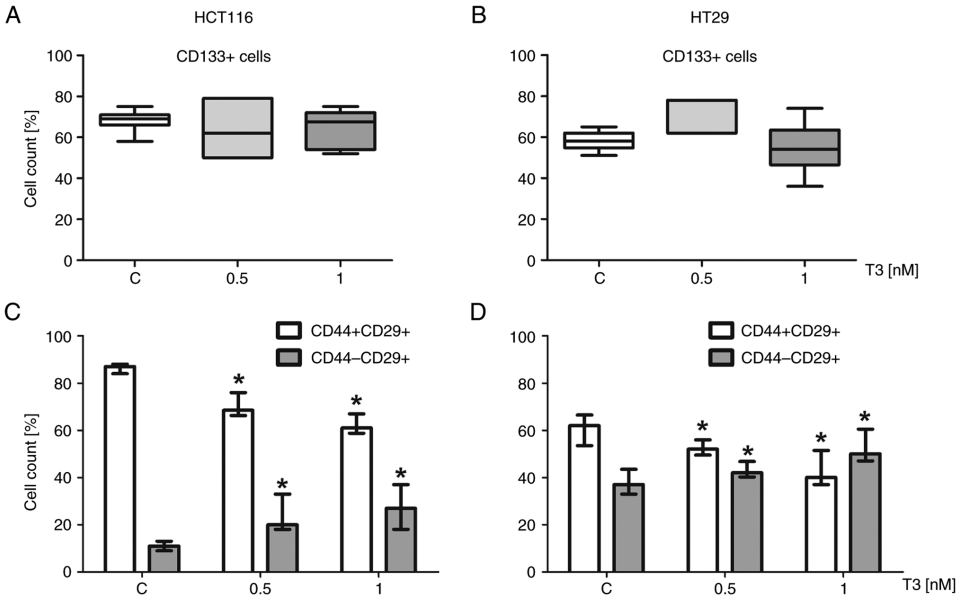

Figure 2. Phenotype of colorectal cancer cell lines expended in a form of colonospheres. The cytometric analysis of colonospheres derived from (A and C) HCT116 and (B and D) HT29 cell lines and treated with thyroid T3 hormone (0.5 or 1 nM). The frequency of cells of given phenotype is presented on Y-axis (%) and compared with untreated control cells. Statistical significance was showed with Kruskal-Wallis test or Mann-Whitney U test. Bars and whiskers represent (A and B) the median with min-max values or (C and D) median ± interquartile range *P<0.05 vs. untreated control cells (C), n=6-12 for each experimental group.

pool (G0/G1) (Fig. 6A-C) of colonospheres obtained from all populations included into the present study. G0/G1 cell cycle growth arrest was observed after the incubation of spheres with T3, while the number of cells in S and G2-M phases (active

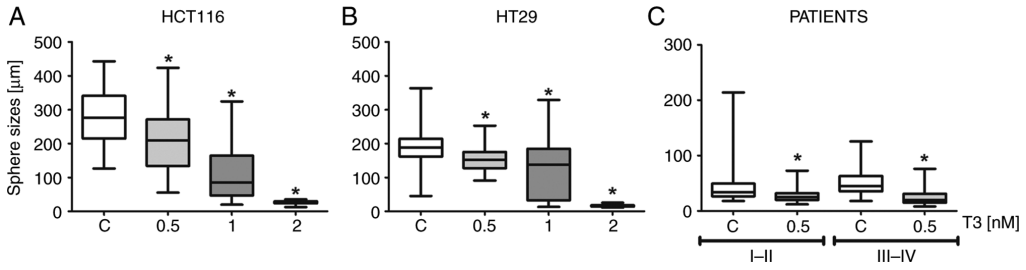

Figure 3. Sizes of colonospheres. Colonospheres were derived from (A) HCT116 and (B) HT29 cell lines, and (C) cancer cells from human CRC tissues. All were treated with thyroid T3 hormone (0.5, 1 or 2 nM) for 3 days. Samples of patients were divided into two groups according to clinical stage of CRC (I-II vs. III-IV). The diameter of at least 50 spheres of each experimental group was measured with an inverted microscope and a digital camera. Statistical significance was showed with Kruskal-Wallis test or Mann-Whitney U test. Bars and whiskers represent the median with min-max values. *P<0.05 vs. untreated control cells (C).

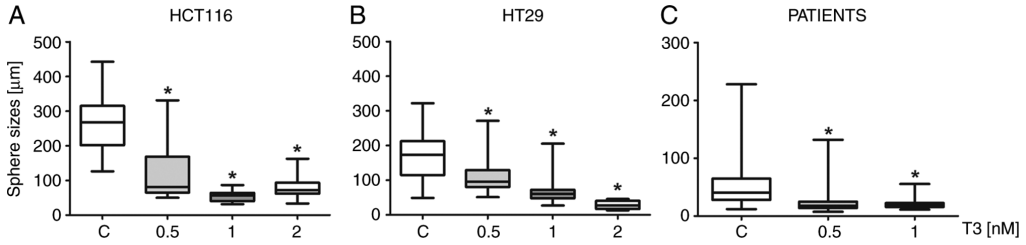

Figure 4. Secondary sphere formation ability. Following the T3 treatment spheres derived from (A) HCT116 and (B) HT29 cells or (C) cells isolated from CRC patients were pooled, suspended and seeded in fresh SCM. Spheres were monitored by measuring the maximal outgrowth of sphere's diameter after 1 week. The diameter of at least 20 spheres of each experimental group was measured with an inverted microscope and a digital camera. Statistical significance was showed with Kruskal-Wallis test or Mann-Whitney U test. Bars and whiskers represent the median with min-max values. *P<0.05 vs. untreated control cells (C).

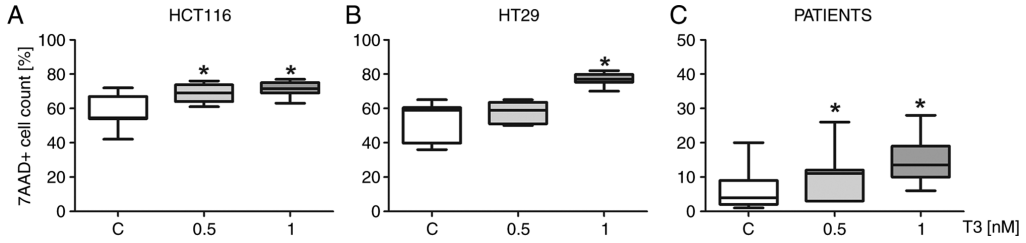

Figure 5. Analysis of cell viability. Colonospheres were derived from (A) HCT116 and (B) HT29 CRC lines and (C) cancer cells isolated from human CRC tissue. They were all treated with thyroid T3 hormone (0.5 and 1 nM). Next, cells were incubated with 7-AAD and analyzed with flow cytometry. Y-axis presents 7-AAD$^+$ cells frequency (%) compared with untreated control cells. Statistical significance was showed with Kruskal-Wallis test or Mann-Whitney U test. Data presented as bars and whiskers representing the median with min-max values. *P<0.05 vs. untreated control cells (C), n=6 for each experimental group.

phases of cell cycle) was markedly reduced in comparison to untreated control.

The viability of CRC cells of HCT116 and HT29 cell lines was analyzed by the assessment of total levels of cellular ATP. The test assumed the decrease of ATP level in samples as an indicator of cell death. The results confirmed that the increased concentration of T3 in culture reduced viability of colonospheres (P<0.05; Fig. 7A). In addition, colorimetric test was performed relaying on MTS reagent to determine the proliferative capacity of HCT116 and HT29-derived colonospheres treated with T3. It was revealed that the proliferation rate was significantly decreased following T3 treatment in comparison to control cells (P<0.05; Fig. 7B).

T3 influences the expression of proteins associated with T3 activity. Since the effects of thyroid hormones depend on the activity of some proteins engaged in their activation or binding, the impact of T3 on protein level of two thyroid hormone receptors (THRα1 and THRβ1) and two deiodinases (DIO2 and DIO3) was evaluated by western blotting.

As demonstrated in Figs. 8 and 9, the results obtained for CRC cell lines-derived colonospheres were not fully consistent

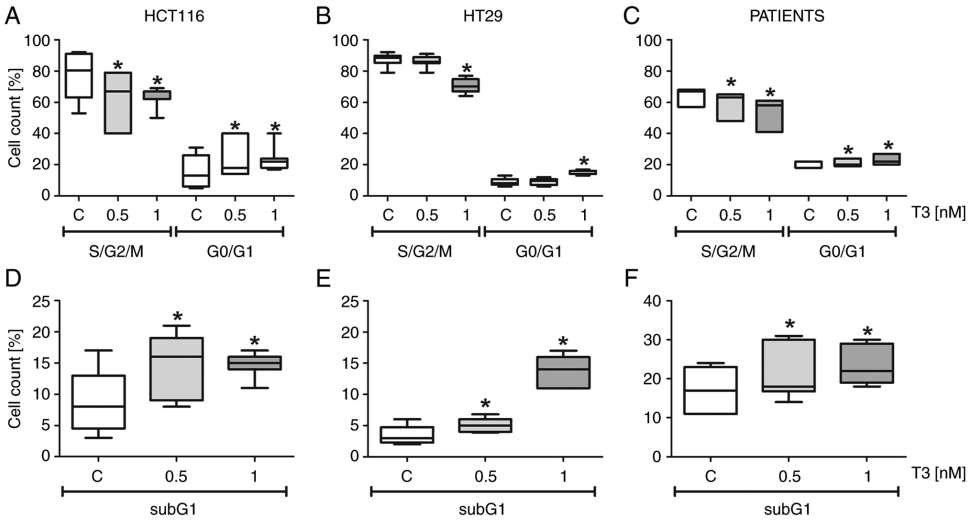

Figure 6. Distribution of cells in cell cycle phases. Cells of colonospheres formed from (A and D) HCT116 and (B and E) HT29 cell lines and (C and F) cancer cells isolated from human CRC tissue treated with thyroid T3 hormone (0.5 or 1 nM) or untreated control cells (C) were analyzed. (A-C) Y-axis presents cell frequency (%) in active (S/G2/M) or in G0/G1 phases or (D-F) dying cells in subG1 phase. Statistical significance was showed with Kruskal-Wallis test or Mann-Whitney U test. Data presented as bars and whiskers represent the median with min-max values. *P<0.05 vs. untreated control cells (C), n=6 for each experimental group.

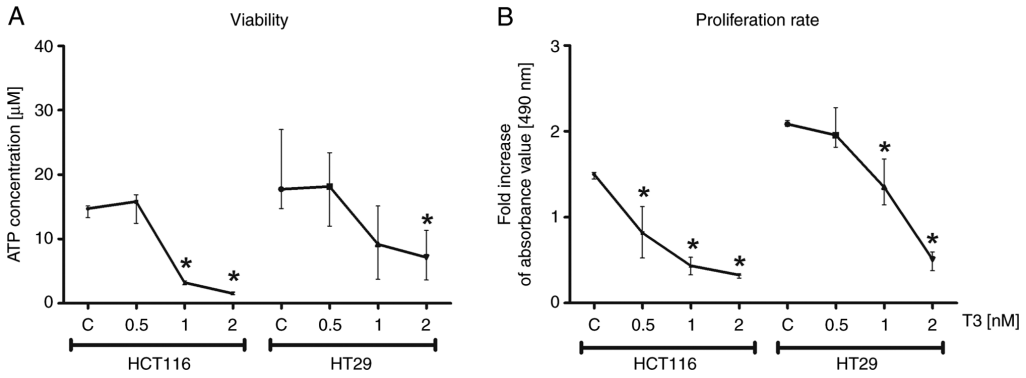

Figure 7. Evaluation of (A) viability and (B) proliferation abilities of colorectal cancer cells. Colonospheres formed from HCT116 and HT29 cell lines were treated with thyroid T3 hormone (0.5, 1 and 2 nM) and analyzed. Statistical significance was showed with Kruskal-Wallis test or Mann-Whitney U test. Bars and whiskers represent median ± interquartile range. *P<0.05 vs. untreated control samples (C), n=6 for each experimental group.

with changes observed in cancer cells obtained from patients. Western blotting of colonospheres originated from HCT116 cells treated with T3 demonstrated increased relative levels of almost all analyzed proteins. The elevations of THRα1 and DIO3 levels were statistically significant in comparison to control (P<0.05). The level of DIO2 remained at the same level after T3 treatment. Concurrently, the expression of all proteins in colonospheres obtained from HT29 cells was lower in treated samples than in control spheres and only DIO3 change did not reach statistical significance. The western blot analysis of colonospheres from patients with CRC revealed no substantial alterations in the expression of proteins assessed in the present study, however, when samples of patients were divided according to cancer clinical stages, the expression of DIOs was found lower in cancer cells obtained from patients with stage III-IV CRC in comparison to samples collected from patients with stage I-II CRC. Furthermore, the DIO2 and DIO3 protein relative levels were mostly upregulated in patients representing group with early cancer (stage I-II).

Discussion

Thyroid hormones play crucial role in the regulation of multiple physiological activities including differentiation, growth and

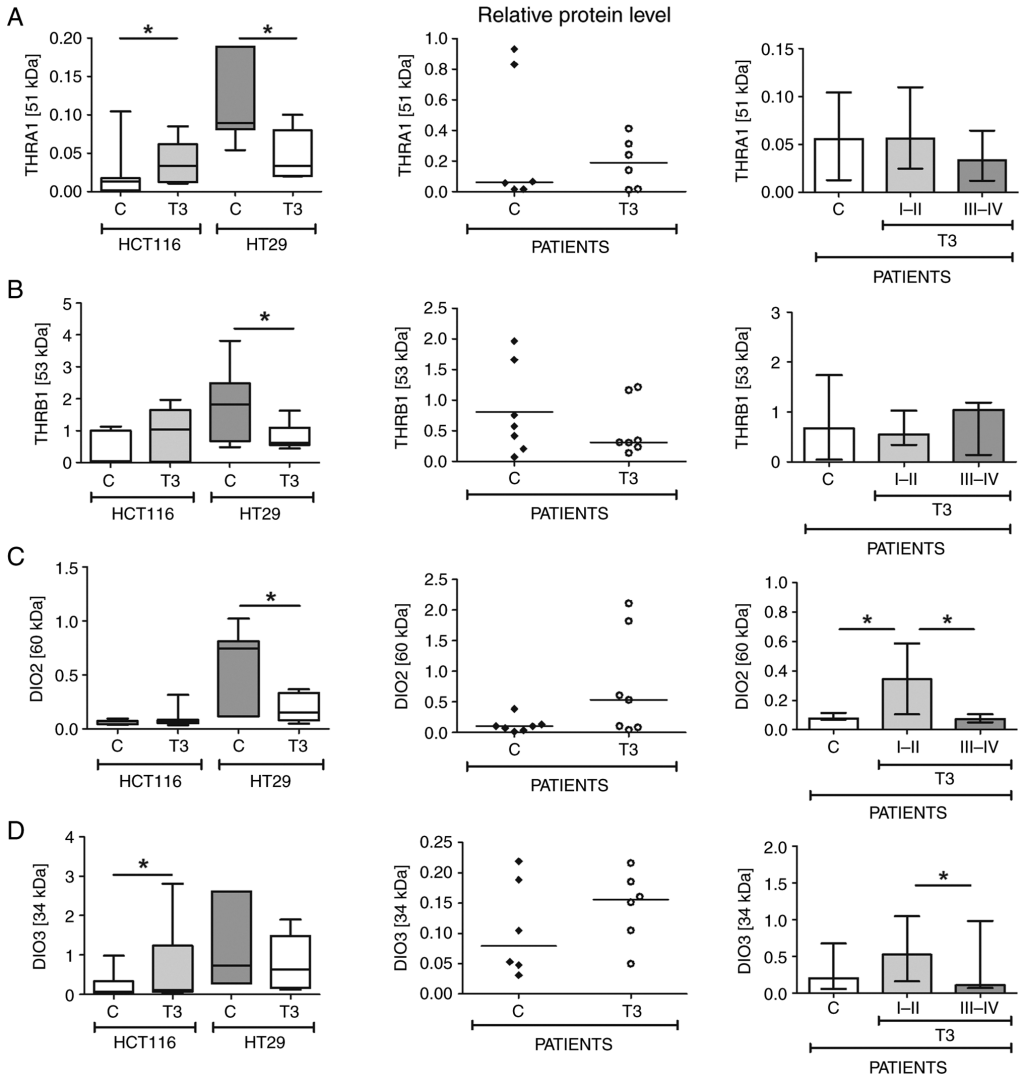

Figure 8. Western blot analysis of (A) THRα1, (B) THRβ1, (C) DIO2 and (D) DIO3. Densitometric analysis was conducted with HCT116 and HT29 cell lines, and cancer cells isolated from human CRC tissue treated with thyroid T3 hormone (0.5 nM) or untreated control cells (C). Additionally, samples of patients were divided into two groups according to clinical stage of CRC (I-II vs. III-IV). Bars and whiskers represent the median ± interquartile range. Y-axis represents relative protein levels determined by densitometric scanning of the bands and corrected for GAPDH loading control. Statistical significance was showed with Kruskal-Wallis test or Mann-Whitney U test. Each experiment was performed from 3-6 times. *P<0.05. THR, thyroid hormone receptor; DIO, deiodinase.

metabolism and are required for normal function of nearly all tissues (18,19). However, thyroid hormones attract attention since their regulatory functions were found crucial for both physiological processes in normal cells of healthy tissues and also have a great impact on the proliferative abilities and cancer growth of cancer cells. Clinical hypothyroidism was found to be associated with delayed cancer development. Concurrently, hyperthyroidism is correlated with increased cancer growth, including breast, thyroid, lung, brain, liver and CRC (1,18,20-22).

The current study provided novel evidence that T3 can be an important modulator of CRC CSC properties. A model of expansion of colonospheres derived from CRC cell lines and CRC cells isolated from cancer tissue was used. These procedures were previously exercised by the authors (10). The experimental conditions mimicked the microenvironment with locally increased T3 concentration where it was monitored how this crucial thyroid hormone altered features of CSCs or colonospheres. It was found that the proportion of $CD133^+CD29^+CD44^+$ stem-like cells was significantly decreased following the 3-day

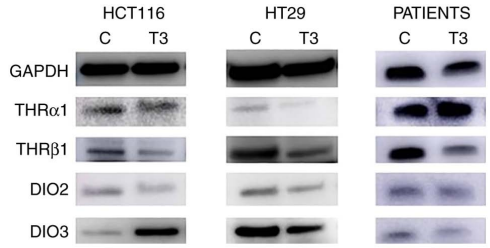

Figure 9. Representative western blot analysis images. Western blot analysis of THRα1, THRβ1, DIO2 and DIO3 proteins in HCT116 and HT29 cell lines and cancer cells isolated from human CRC tissue treated with thyroid T3 hormone (0.5 or 1 nM) and compared with untreated control cells (C). Presented sets of bands were obtained in the same western blot membrane. THR, thyroid hormone receptor; DIO, deiodinase.

treatment with T3, suggesting that treated colonospheres displayed a lower content of CSCs in comparison to untreated control. Indeed, it was identified that T3 significantly inhibited the ability of cancer cells to form colonospheres by increased apoptosis and elevation of G1/G0 silenced pool within cultures. Similarly, the treatment of MCF breast cancer cell line with T3 resulted in decreased proliferative and migratory potential of these cells and reduced number of CSCs (23).

The actions of T3 are initiated by binding to nuclear thyroid receptors (TRs), encoded by two genes, α and β, which give rise to different receptor isoforms (24). TRs are widely distributed in mammalian tissues, but transformed or immortalized cells in general express very low levels of TRs. In addition, there is increasing evidence that alterations in TRs are common events in cancer and it has been suggested that TR genes may function as tumor suppressors (25-28), although the role of these receptors in the pathogenesis and progression of neoplastic processes is currently unclear (28) and results in different types of cancer are not fully consistent (20,22,29-32). The present results are concomitant with this general concept since lower THRα1 and THRβ1 relative protein levels were observed in untreated colonospheres derived from HCT116 cell line which represent CRC with higher progression status in comparison to HT29 cells (TNM3/Dukes' D vs. TNM2/Dukes' C, respectively; $P<0.05$) (33). Tissue samples of patients presented more diverse results which can be partially explained by heterogeneity of individuals recruited to the present study. CRC cells appeared to sustain both receptors at low but constant level for the potential need to activate the expression of T3 target genes (34). Similar results concerning expression of thyroid hormone receptors were described by Wang *et al* (2), who reported that thyroid hormones increase cells' self-renewal capacity and the percentage of $CD90^+$ CSCs and promote drug resistance of primary human HCC cells. It was suggested that THRβ1 acts as tumor suppressor in a number of cancers and one of the proposed mechanism relay on upregulation of the nuclear receptor co-repressor 1 and suppression of invasion, tumor growth, and metastasis in human as it was demonstrated for hepatocellular carcinomas (35) and neuroblastomas (36). In addition, breast cancer mammospheres presented reduced tumorigenesis upon stimulation of THRβ1 (37). The possible mechanisms are downregulation of cyclin D1 expression and modulation of the TNFα-NFκB signaling (23,37). Certain of the present results supported these previous observations since it was observed that the expression of THRβ1 receptor was significantly lower in HT29 cells following T3 treatment. In addition, an increased level of THRβ1 protein was demonstrated along with clinical advancement of CRC.

The primary secretory product of the thyroid gland, 3,5,3',5' tetra iodothyronine (T4) or thyroxine, must be converted to T3 for its activation. DIO2 (activating DIO) converts the prohormone thyroxine to the active thyroid hormone T3, whereas DIO3 (deactivator DIO), by inactivating both T4 and T3, terminates thyroid hormone action (38). This pre-receptor process is an essential mechanism that regulates thyroid hormone function at the intracellular level. In colorectal adenomas and carcinomas, DIO3 expression is significantly higher than in normal tissues and negatively correlates with the histologic grade of the lesions suggesting that DIO3 could be a marker of early stages of carcinogenesis (38). That appears to be consistent with the present results conducted with both CRC cell lines and cells of patients. Higher DIO3 level was observed in samples derived from patients with lower CRC stage (I-II) in comparison to patients with III-IV stage. The parallel increased expression of both DIOs in cancer cells was assumed to rapidly customize the thyroid hormone signal if necessary what was previously presented for BCC cells as well (39). The alterations in protein levels of both enzymes depended on cancer clinical stage and it was supposed to protect cells from anti-proliferative and anti-survival effect of T3 *in vitro* cultures. Similar conclusions may be drawn from the analysis of levels of DIOs in the samples of the present study. Higher DIO2 and DIO3 was revealed in stage I-II CRC samples compared with untreated control and stage III-IV CRC cells. These results are in accordance with observations of increased stromal DIO2 level of intestinal polyps of ApcΔ716 mice, a mouse model of familial adenomatous polyposis and early stage sporadic CRC (40).

Furthermore, it was identified that DIO3 is a direct transcriptional target of the β-catenin/TCF complex and its expression was higher in human intestinal adenomas and carcinomas than in healthy intestinal mucosa. Experimental attenuation of β-catenin reduced DIO3 levels and induced DIO2 thereby increasing T3-dependent transcription. In the absence of DIO3, the T3 excess reduced cell proliferation and promoted differentiation in cultured cells and in xenograft mouse models (38). Increased level of DIO3 in samples after treatment with T3 may be an evidence that the overabundance of active thyroid hormone can compromise the fragile niche homeostasis. The increasing T3 concentration inhibited proliferation of treated cells which can be an effect of insufficient DIO3 level. The decline was more significant for HCT116 cells with lower initial DIO3 expression. The CSCs tended toward silencing their activity after treatment with elevated T3 concentration which was revealed with the analysis of phenotype and cell cycle.

It was also observed that the incubation of colonospheres with T3 decreased viability, proliferative and spherogenic potential of cancer cells. In addition, increased apoptotic rate of CRC cells was revealed. Concurrently, cells of treated colonospheres were more likely to move into silenced pool in G0/G1 phase of the cell cycle. Additionally, the smaller sizes of colonospheres following the treatment with T3 suggested that T3 can lower the proportion of primitive cells actively supplying

the pool which proliferate within spheres. It was hypothesized that this could be a protective mechanism aimed to avoid the elimination of all cancer-initiating cells from colonospheres. In this context it was confirmed that the hormonal constituents of cancer niche have a crucial role for the fate of CSCs.

The technical limitation is the use of 1.0 N NaOH-based solvent to prepare T3 solutions. Although our initial study demonstrated that the buffer did not influence cells features, the use of clear medium to dissolve T3 powder may have provided more *in vivo*-like results.

The authors are conscious that the present study presents only a part of cancer-related interactions between certain micro-niche components. The analysis of a narrow fragment of thyroid hormone homeostasis was conducted, rather than the whole TSH-T4-T3-rT3 axis. These hormones may be considered biologically active for healthy cells; however, more efforts are needed to evaluate the role of T4 and T3 in cancer cells. One of the limitations of the present study was the insufficient number of patients to correlate some more clinical parameters with obtained laboratory results. Individuals with CRC are a heterogeneous group of patients, for instance, according to the location of the disease. Cancers localized in rectum and colon are biologically different. All criteria could not be included because our study groups would be too small and subsequent analyses of subsets would be underpowered. Exclusion of patients after neoadjuvant chemoradiation renders comparisons easier and more homogenous but it does not analyze the real-life scenario. Particularly that certain hormones and drugs are known to have a specific interplay with chemotherapy or radiosensitvity. Nevertheless, the present study proposed a novel function of thyroid hormone signaling in the regulation of CRC CSCs fate and the following projects by the authors may extend the patients group to analyze the effect of other clinical parameters on thyroid hormone axis in CSCs in CRC.

In conclusion, considering the present results, it could be hypothesized that thyroid hormones exert a great impact on the fate of colorectal CSCs. Since CRC is one of the most prevalent cancers worldwide the unveiling novel aspects of CRC niche seems to be extremely important. It was found that the T3 role in cancer biology depends on clinical stage of CRC and in near future such data may be taken into consideration in therapeutic decision making. Proteins involved in thyroid hormone homeostasis by their concerted actions in cancer niche cooperate in the synchronized manner with cellular internal elements associated with next steps of carcinogenesis. However, the signals regulating functions of all these elements during cancer development are largely unknown. CSCs which are considered to be responsible for the most serious events in carcinogenesis were found to be highly sensitive to T3. The cellular balance of thyroid hormone associated proteins can be considered as a potential therapeutic target for designing anticancer drugs. The results of the present study suggested that thyroid hormone homeostasis may have potential prognostic and therapeutic value in human CRC. Further research evaluating the detailed functions of thyroid hormone axis on molecular level and their exact biological mechanism may enable introduction of specific chemotherapeutic protocols in the future.

Considering that T3 was found to target CSCs it can be considered to be supplemented to CRC patients to influence the content of tumor microenvironment. Previous studies demonstrated that depletion of endogenous T4 to increase T3 can improve clinical outcomes of patients with advanced cancer. In patients with pre-existent primary hypothyroidism, T3 administration was observed to lower the T4 level and improve survival of patients (4,41). The adjunctive therapy with thyroid hormones can give a hope to improve the efficacy of conventional chemotherapy, however, analyses concerning different type of cancer arises numerous controversies and further research is needed.

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Availability of data and materials

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

OR, MS, PS and JK conceived the study. OR, MS, AO-K, PS and JK developed methodology. OR, MS and JK performed software analysis. OR, MS and JK validated the data. OR, MS, AO-K and PS performed formal analysis. MS, AO-K and PS conducted investigation. MS provided resources. RO, MS and AO-K curated the data. RO and MS prepared the original draft. AO-K, PS and JK wrote reviewed and edited the manuscript. MS and JK supervised the study. JK acquired funding. All authors have read and approved the final version of the manuscript. OR, AO-K, MS, PS and JK confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The present study was conducted according to the guidelines of the Declaration of Helsinki, and was approved (approval no. NKBBN/203/2020) by The Independent Bioethics Committee of Medical University of Gdansk (Gdansk, Poland) from 24.04.2020. Informed consent was obtained from all subjects involved in the present study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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