INVESTIGATION OF GENETIC AND MOLECULAR DETERMINANTS OF THYROID CANCER HETEROGENEITY AND PROGRESSION

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TABLE OF CONTENTS

1. Abstract/Résumé

Thyroid cancer is the most common endocrine malignancy, frequently among young adults and women. The most common subtype of thyroid cancer is papillary thyroid carcinoma (PTC), a well-differentiated and relatively indolent malignancy. However, some PTCs show aggressive behaviour with a high incidence of metastasis and recurrences. The overall objective of this research is to identify biomarkers that can discriminate between non-aggressive and aggressive PTC types. The first part of this work was to profile genomic alterations that occur in metastatic and non-metastatic PTC cases and to identify those that can serve as molecular signatures for aggressive forms of PTC. For this purpose, deep sequencing analysis on RNA isolated from fresh PTC tissues was carried out. Over 1000 genes differentially expressed in metastatic samples were identified, among which RSPO4 gene was specifically overexpressed in BRAF V600E mutated PTC. The second part of this research was to investigate independent factors that may impact on PTC development in general, focusing on vitamin D3, which has been investigated previously to regulate both genetic and epigenetic events and to exert a protective effect on PTC in some case-control studies. A meta-analysis was conducted to document that host-related genetic and epigenetic determinants associated with vitamin D signalling could greatly impact on individual response to vitamin D and may outweigh the conflicting outcome of previous case-control studies using classical endpoints such as sun exposure or vitamin D dietary intake. The final part of this thesis works to explore the impact of a novel small molecule identified to interfere with cell signalling associated with cell differentiation programming on differentiation and tumorigenic features in an anaplastic thyroid carcinoma (ATC) preclinical model. ATC malignancy remains one of the most aggressive and deadly human malignancies overall. In this context, a small molecule capable of targeting anaplastic thyroid cancer cells *in vitro* and *in vivo* when transplanted into RAG mice was identified.

Résumé

Le cancer de la glande thyroïde est le cancer le plus fréquent touchant un organe du système endocrinien et se présente fréquemment chez les jeunes adultes et les femmes. Le type le plus commun de cancer de la glande thyroïde est appelé carcinome papillaire et est une néoplasie relativement indolente. Pourtant, certains carcinomes papillaires montrent un comportement plus agressif avec un haut taux de métastases et de récidives. L'objectif de cette thèse est d'identifier des biomarqueurs capable de discriminer les carcinomes papillaires agressifs des carcinomes papillaires indolents. La première partie de la thèse se concentre sur la caractérisation des

altérations génomiques occurrentes dans les carcinomes papillaires métastatiques. Un séquençage de l'ARN de tumeurs fraiches a été effectué plus de 1000 gènes montrant une expression différente entre les cas de carcinome papillaire métastatique et non-métastatique ont été identifiés. La deuxième partie de la thèse avait pour but d'examiner les facteurs contribuant au développement de carcinome papillaire de la thyroïde, en particulier la vitamine D. La vitamine D a été impliquée dans des études précédentes comme facteur protecteur abaissant le risque du cancer de la thyroïde. Dans une méta-analyse, il a été montré que des facteurs génétiques impliqués dans la régulation de la vitamine D influe de manière déterminante l'effet biologique de celle-ci et que les études mesurant une simple association entre les niveaux de vitamine D dans le sang/ou l'exposition au soleil ont probablement une validité limitée. La partie finale de la thèse consiste à explorer l'efficacité d'une nouvelle molécule chimique capable d'induire une reprogrammation des cellules cancéreuses dans le cancer anaplastique de la glande thyroïde. Le cancer anaplastique de la thyroïde est une des formes les plus létales de cancer chez les humains. Dans ce contexte, une molécule montrant une efficacité antinéoplastique in vitro et in vivo a été identifiée et caractérisée.

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Last but not least

Isabel

3. Preface and Contribution of authors

Thesis writing: GB Morand

Manuscript 1:

Authors contributions

MA Alaoui-Jamali, SD da Silva and MP Hier were the project leaders. GB Morand performed under their supervision the vast majority of the experimental work reported. RNA Extraction was carried out by GB Morand, SD da Silva and Dinghong Qiu. RNA sequencing and bioinformatics analysis done at McGill University Innovation Centre-Genome Quebec. Validation qPCR done by GB Morand and SD da Silva. Cell based assays were done by GB Morand under A Saad and SD da Silva's guidance. Manuscript drafting and figures were done by GB Morand under MA Alaoui-Jamali, SD da Silva and MP Hier's supervision. All the authors have participated substantially to the final manuscript and approved the final version.

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Authors contributions

GB Morand performed the literature search, the retrieval of the studies, the data extraction and wrote the main part of the manuscript under SD da Silva and MA Alaoui-Jamali's supervision. All the authors participated substantially to the final manuscript and approved the final version.

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Authors' contributions

GB Morand conducted the in vitro experiments (excepted for the FACS analysis done by H Yu), did the in vivo experiments with help of J Su, constructed the figures, wrote the manuscript under supervision of K Bijian and MA Alaoui-Jamali. D Wernic synthetized the chemical compounds under K Bijian and MA Alaoui-Jamali's supervision. All the authors participated substantially to the final manuscript and approved the final version.

3.1. Claim of Originality

This thesis is the first to investigate, using three different approaches, the genetic and molecular determinants of thyroid cancer heterogeneity and progression. The first part is, to date, the first study to compare using deep sequencing the genomic profile of metastatic and non-metastatic thyroid cancer. In the second part, the first comprehensive and systematic review on thyroid

cancer and vitamin D was performed, with the impact of genetics on a putative association between the former and the latter. The final part proves for the first time the antineoplastic activity of a novel purine derivative chemical in the most lethal from of thyroid cancer, namely anaplastic thyroid carcinoma.

4. Rationale and objectives

Follicular cell-derived thyroid cancer is a common malignancy, particularly in young females patients, that usually behaves indolently. However, some less common thyroid cancer subtypes show aggressive phenotypes with higher rates of metastasis and recurrence. The discrepancy seen in the same "conceptual" entity of thyroid cancer can be explained by host and cancer-related factors. Specifically, both the genetic background of the patients and the molecular features of the tumor itself determine the phenotypic and clinical behaviour of the malignancy.

The specific objectives of this thesis were to:

Manuscript 1: Compare genomic expression in metastatic vs. non-metastatic thyroid tumors using high-throughput technology (RNA Seq. Our goal was to identify and validate a novel biomarker that can predict TC aggressive phenotypes.

Manuscript 2: Document, using the example of vitamin D and TC development, that individual genetic variations are critical determinants in the evaluation of potential preventive and anticancer properties of specific agents in TC.

Manuscript 3: Evaluate a novel therapeutic approach for poorly differentiated and highly aggressive forms of TC.

5. General introduction with literature review

5.1. Overview of thyroid cancer and histological subtypes

Thyroid cancer is the most common endocrine malignancy worldwide [1]. This tumor is classically divided into four subtypes based on histological features: papillary (PTC), follicular (FTC), medullary (MTC) and anaplastic (ATC) [2]. Among these subtypes, PTC is the most frequent,. representing 70-85% of all thyroid cancer cases in North America [3, 4]. PTC and FTC are derived from follicular cells and show similar biologic behaviour. They are often grouped together as well-differentiated thyroid carcinoma (DTC) as opposed to poorly differentiated (PDTC) and ATC [5]. PDTC is considered an intermediate stage between DTC and ATC. ATC is one of the most aggressive human tumors with close to 100% disease-specific mortality [6]. It is thought to result from progressive dedifferentiation of DTC [7, 8], either via the classical multifactorial and multistage carcinogenic process [9], or via the new concept of precursor cells with cancer stem cell-like properties [10].

5.2. Epidemiology of thyroid cancer

Epidemiological studies have reported an increase in the incidence of DTC particularly among young patients and women [3, 11-18]. Known risk factors include exposure to radiation [19-22] and inherent genetic factors related to family history and ethnicity [17, 23, 24]. The cause of the rising incidence of TC is not understood but factors such as overdiagnosis of subclinical disease have been proposed as a result of increasing wide access to health care [25], although overdiagnosis alone does not fully explain this increase [26]. Further risk factors have been investigated, including volcanic area chemical exposure [27-29], obesity [30-33], iodine deficiency [34] or excess [35], and Hashimoto thyroiditis [36].

While most previous studies have focused on risk factors for TC, some reports also identified protective factors, such as high vegetable intake, green tea, trace elements or vitamin intake [37-40]. Among these, growing evidence reports the potential chemopreventive role of vitamin D in thyroid cancer (Manuscript 2).

5.3. Aetiology and molecular basis of thyroid cancer

Recent studies have led to progress in understanding the molecular mechanisms in DTC [41-44]. The activation of the MAPK and PI3K-AKT signalling pathways [45], common mutations of the BRAF and RAS genes as well as RET/PTC and PAX8/PPARγ chromosomal rearrangements [46-53] have been well described. These mutations are investigated as potential diagnostic tools in

clinical practice, mainly to help clinicians in the management of patients with thyroid nodule and in distinguishing benign from malignant disease [41, 42, 48, 54-56]. However, molecular mechanisms leading to metastasis in thyroid cancer are poorly understood [57, 58].

A key element in tumor progression is introduced through the concept of epithelial mesenchymal transition (EMT). EMT is a multi-step and reversible process mediated by specific molecular signals that promote the loss of cell–cell junctions, cell–matrix adhesion, and reorganization of the cytoskeleton. This results in loss of the apical polarity associated with epithelial cells and gain of mesenchymal characteristics such as spindle-shaped morphology and a high degree of motility [59-63]. EMT also increases the proportion of cancer stem cell (CSC) subpopulation within a tumor, thereby generating self-renewing cells that are both tumorigenic and believed to be intrinsically resistant to chemo- and radiation therapies [64-66]. Many growth factors and cytokines essential to the regulation of EMT phenotype and secreted in an auto-/paracrine manner in tumor microenvironment have been identified, e.g. TGFb, epidermal growth factor (EGF), IL6 and TNFa [67-73]. Few studies investigated specifically EMT and thyroid cancer and most focused on EMT and ATC [7, 70-80]. Therefore our aim was to address this aspect in details using clinically relevant cohorts of clinical samples and preclinical models (the later implying both cancer cells and mouse models).

5.4. Treatment of thyroid cancer

Although DTC is almost always curable, management for DTC remains controversial. The natural history and recurrence rates differ gretly among patient subgroups [81-83] thus impeding the development of standard treatment protocol. In low-risk patients, total thyroidectomy might result in overtreatment, and some authors suggest that lobectomy or even surveillance can be sufficient [84]. Some high-risk DTC patients show up to 36% lymph node metastasis rates [85] and up to 30% locoregional recurrence rates [86-88], requiring a more aggressive treatment.

While surgical removal of macroscopic lymph node metastasis is not a matter of discussion, the management of clinically negative locoregional disease is heavily debated. Surgery can potentially lead to a higher morbidity and the benefit remains controversial [89-97]. Clinically negative regional disease tends to be “minimized” for instance in European countries where the use of radioiodine ablation therapy (RAI) is more widespread [98].

5.5. Prognosis of thyroid cancer

Prediction tools exist that take into account several clinical features to distinguish indolent from aggressive DTC. Besides the classical TNM stage, AMES [99], AGES, and MACIS are scoreing tools for this classification [100, 101] (AMES= Age, distant Metastasis, tumor Extension and Size; AGES= Age, Grade, Extension, Size; MACIS: distant Metastasis, Age, Completeness of surgery, Invasion and Size). These tools integrate known negative predictive factors in DTC, such as histological subtypes [102, 103], tumor size, extra-thyroidal extension [104], age greater than 45 at diagnosis, male gender [105], multifocality [106], and regional and distant metastasis [107]. Studies comparing the prognostic utility of different systems reported a relatively low proportion of variance explained (PVE) and high variability among studies [108], thus emphasizing the need for more reliable molecular markers.

6. Manuscript 1: Functional characterization of a pro-metastatic RSOP4/B-Raf signalling loop identified through a deep sequencing analysis of progressive papillary thyroid cancer

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6.1. Abstract

Introduction: Papillary thyroid carcinoma (PTC) is by far the most common malignancy of the thyroid gland and early stages are generally indolent. However, some PTCs show an unusually more aggressive phenotype with extensive lymph node metastasis and higher incidence of locoregional recurrence. Activating mutations of the BRAF kinase (V600E) and expression of epithelial-mesenchymal transition (EMT) phenotypes have been proposed among drivers of PTC aggressiveness but molecular mechanisms behind this association are not fully established.

Methods: We conducted a high-throughput RNA sequencing (RNA Seq) to investigate molecular profiles of PTC tissues from a cohort of patients diagnosed with metastatic and non-metastatic PTC. We further examined genes specifically deregulated in BRAF mutated PTC and their implication for metastasis signalling.

Results: We identified 1045 of 17380 genes to be differentially expressed between metastatic and non-metastatic PTC tumors. Validation by qPCR confirmed several EMT associated-genes (e.g. FN1, CTNNA2, CDH6, MMP7) to be overexpressed in metastatic compared to non-metastatic tissues. Further analysis revealed that all BRAF-mutated (V600E) PTC (but not BRAF-wild-type PTC) tumors present an overexpression of RSOP4, a member of the RSOP family involved in the regulation of WNT signalling. Basic studies in PTC cell lines further support that BRAF V600E mutation can promote activation of RSOP4 and WNT pathway.

Conclusion: Our results support that cooperative signalling between BRAF and WNT pathways involving RSOP4 contribute to PTC aggressiveness.

6.2. Introduction

Thyroid cancer is the most common endocrine malignancy worldwide [1]. Among different histological types, papillary thyroid cancer is the most frequent representing 70-85% of all thyroid cancer cases in North America [3, 4]. Epidemiological studies have reported an increase in PTC incidence, particularly among young patients and women [3, 11-18]. Known risk factors include exposure to radiation [19-22] and inherent genetic factors related to family history and ethnicity [17, 23, 24]. Although PTC is almost always curable, controversies exist in the management of PTC, as the natural history and recurrence rates greatly differ among patients' subgroups [81-83]. Some high-risk PTC patients show up to 36% lymph node metastasis [85] and up to 30% recurrence [86-88], requiring a more aggressive treatment.

Rapid advances in transcriptome analysis technologies allowed for exploration of factors driving tumor aggressiveness and response to therapy [41-44, 109]. Mutations in key protooncogenes are found in about 70% of PTC. Among them, the V600E mutation in BRAF is the most common but has been inconsistently associated with aggressive clinical manifestations and poor prognosis [46, 48, 110-112]. Furthermore, acquisition of the epithelial-mesenchymal transition (EMT) phenotype has been implicated in progression of thyroid cancer to metastasis [7, 70-80]. EMT is a reversible process involving profound changes involving loss of cell-cell junctions and cell–matrix adhesion, and associated with overexpression of several transcriptional repressors of E-cadherins, including Twist, Snail, and Slag transcription factors. This results in a loss of the apical polarity of epithelial cells and gain of mesenchymal characteristics such as spindle-shaped morphology and a high degree of motility [59-63]. EMT also increases the proportion of cancer stem cell (CSC) subpopulation within a tumor, thereby generating self-renewing cells believed to drive both tumorigenesis and metastasis, as well as to contribute to chemo- and radiation relapses [64-66].

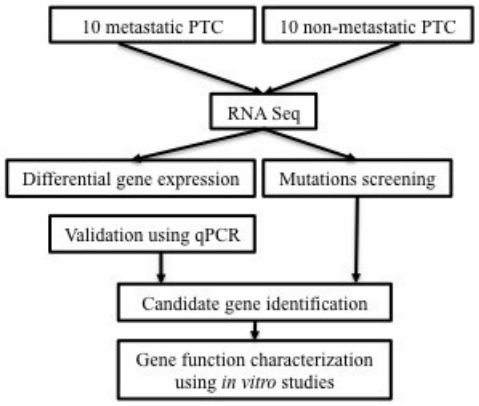
In the current study, we conducted a detailed RNA sequencing (RNA-Seq) in order to investigate genetic changes associated with aggressive PTC using clinical metastatic versus non-metastatic papillary thyroid tumors. We identified metastatic tumors with BRAF V600E mutation to overexpress R-spondin 4 gene, an activator of WNT signalling.

6.3. Materials and Methods

Participants

Overview of the analytical approach is shown in Figure 6.1. After Ethics Review Board approval (#13-093), we included papillary thyroid cancer patients prospectively enrolled in the fresh tissue biobank of Sir Mortimer B. Davis-Jewish General Hospital in Montreal, QC, Canada. Patients were consented at the time of surgical booking for use of their pathological fresh frozen and formalin fixed paraffin embedded samples. Furthermore, full access to their medical records was granted for research purposes. Detailed demographics (clinical stage, lymph nodes involvement, histological subtype and variant, extrathyroidal extension, multifocality, and surgical margins), treatment, follow-up and recurrence data were obtained retrospectively. Clinicopathological data were handled in a coded fashion according to ethical guidelines. Exclusion criteria were papillary micro-carcinoma, pregnancy, age less than 18 years, and missing follow-up.

Figure 6.1: Samples and experimental design.



Sample preparation and RNA isolation

We used a set of RNA samples from 20 fresh-frozen thyroid cancer samples and 4 morphologically normal tissues from thyroid. Fresh-frozen tumor material was collected at the time of surgical resection, snap-frozen in liquid nitrogen, and stored at -80°C until RNA extraction. Time between tumor resection and storage in liquid nitrogen did not exceed 45 minutes. Metastatic samples were matched for age, sex, and T category to assure comparability of the two groups (Table 6.1). RNA extraction was done using mRNeasy Kit (Qiagen, Inc, CA) according to the manufacturer's instruction.

Table 6.1: Clinico-pathological characteristics of RNA Seq patients.

Group ***Variable***	**Non-metastatic (N=10)**	**Metastatic (N=10)**	***P* value***
Histological subtype	10/10 papillary	10/10 papillary	0.99
Age (mean, SE)	45.9 (4.7)	46.3 (4.8)	0.95
Gender (f/m)	7/3	6/4	0.99
pT category (T3/T2-1)	4/6	4/6	0.99
pN category	pN0 (10/10)	pN+ (10/10)	<0.001

*Fisher's exact for binary variable; t-test for continuous variable.

RNA Quantification and Quality Control

All RNA samples were assessed for quality using the RNA 6000 Nano assay on the 2100 Bioanalyzer (Agilent Technologies, Inc) and for quantity by Nanodrop (Thermo Scientific). Suppl. Fig. 1 (see section 6.7), Panel A depicts typical Agilent Bioanalyzer profiles seen upon submission of clinical samples. All samples had to pass quality control to be further used. In total, 37 extractions of RNA from fresh frozen samples were necessary to obtain high quality RNA for 20 samples.

RNA Library Construction, Sequencing

rRNA depletion was performed on 200-400ng of each total RNA sample with the RiboZeroGold (Illumina, San Diego, CA) as per the manufacturer's instructions. The entire rRNA depleted fraction (ranging 4-22ng) was used as input for library preparation using the ScriptSeq V2 library preparation kit (Illumina, San Diego, CA). All libraries were validated and quantified with the Bioanalyzer DNA 1000 assay (Agilent Technologies, Inc, CA) and further quantified with the Qubit DNA Broad Range assay (Life Technologies, Carlsbad, CA). 10µL of each library were diluted to a concentration of 10nM. Equal volumes of each 10nM library were then pooled for subsequent paired-end sequencing on an Illumina HiSeq 2000/2500 (Illumina). Sequencing was performed with 4 samples per lane, hence generating 62 to 106 million paired reads per library. Base calls were made using the Illumina CASAVA pipeline. Base quality was encoded in phred

33. Usable read count for each sample is shown in Suppl. Fig 1, Panel C, while Panel B depicts mean coverage from 3' during sequencing (see section 6.7).

Bioinformatics' analysis

Sequence alignment and quantification of exon expression was carried out using an internally developed RNA-Seq analytical pipeline at McGill University (Suppl. Fig 2, reproduced with permission, Section 6.7). Reads were trimmed from the 3' end to have a phred score of at least 30. Illumina sequencing adapters were removed from the reads, and all reads were required to have a length of at least 32. Trimming and clipping were done with the Trimmomatic software[113]. The filtered reads were aligned to a reference genome (hg19). The alignment was done with the combination of tophat/bowtie software[114]. Trimming and alignment statistics for each sample are shown in Suppl. Table 1 (Appendix). The Cufflinks program [115] was used to assemble aligned RNA-Seq reads into transcripts and to estimate their abundance (FPKM). Several metrics and exploratory analysis in order to control the data quality and to verify the biological reliability of the data were used and are presented in the first sections of the results. The differential gene expression analysis was done using DESeq[116] and edgeR[117] R Bioconductor package. Details about analysis parameters and software versions are available in Suppl. Table 2 (Appendix). RNA library construction, sequencing and bioinformatics analysis were performed at the McGill University and Genome Quebec Innovation Centre (see acknowledgements).

Cell lines, expression plasmids and transfection

The cell lines 8505-c, BCPAP, and PTC1 were a courtesy of Dr. M. Trifiro (McGill University) [118]. Furthermore, we used HEK293 cell lines to test out transfections. These cells were maintained in culture in RPMI or DMEM medium supplemented with 10% heat-inactivated foetal bovine serum (Mediatech®) and 50U/mL of penicillin-streptomycin (1%). A pCMV6-Entry plasmid for *RSPO4*, transcript variant 1, was bought from OriGene®. After amplification, this plasmid was transfected in order to overexpress the RNA and protein of interest, respectively. Transfections were done using Optimem, Polyethylenimin (PEI) and 1mg of DNA in accordance with the manufacturer's protocol.

Real-time PCR

RNA was extracted using using mRNeasy Kit (Qiagen®) according to the manufacturer's instruction. cDNA was synthesized from 500ng of RNA using Superscript II reverse transcriptase

(Invitrogen®) and oligo-dT primers (Invitrogen®). qRT-PCR was be performed in the ABI PrismTM 7900 (Applied®) using SYBR® Green (Applied®) in a 10µL total volume and quality controls were used as proposed by MIQE Guidelines [119]. Suppl. Table 3 provides overview and sequences of used primers. The reactions were carried out in triplicate. *HRPT1* was used as endogenous control. Fold differences in the relative gene expression were calculated using Pfaffl model[120]

Western blot analysis

Total cell extracts from exponentially growing cells were collected by scrapping into modified radioimmunoprecipitation assay lyses buffer supplemented with 20mg/mL pepstatin A, 1mM PMSF, and protease inhibitor cocktail (Roche). Blots were detected using specifics antibodies in accordance with the manufacturer's recommendations and the signal was detected with peroxidase–conjugated secondary antibodies and enhanced chemiluminescence detection system. When indicated, membranes were subsequently stripped for reprobing with internal controls such as GAPDH or a-Nucleolin.

***In vitro* motility and invasion assays**

Cell invasion assays were performed using 8µm porous chambers coated with Matrigel (BD Biosciences ®) according to the manufacturer's recommendations. Cell migration were assayed using the qualitative wound-healing assay. Each experiment was performed at least three times.

Statistical analysis

For dichotomous variables, the two-sided Fischer Exact test was used to compare proportions. Odds ratio (OR) and 95% confidence interval (95%CI) were calculated according to the Mantel-Haenzel method. For discrete variables showing normal distribution, means and standard errors of means (SEM) are given and comparisons were made using the t-test. Alternatively, median, first quartile (Q25), and third quartile (Q75) are indicated and the non-parametric Kruskal-Wallis test was used. Statistical analyses were performed using SPSS® 21.0.0 software (IBM©, Armonk, NY, US).

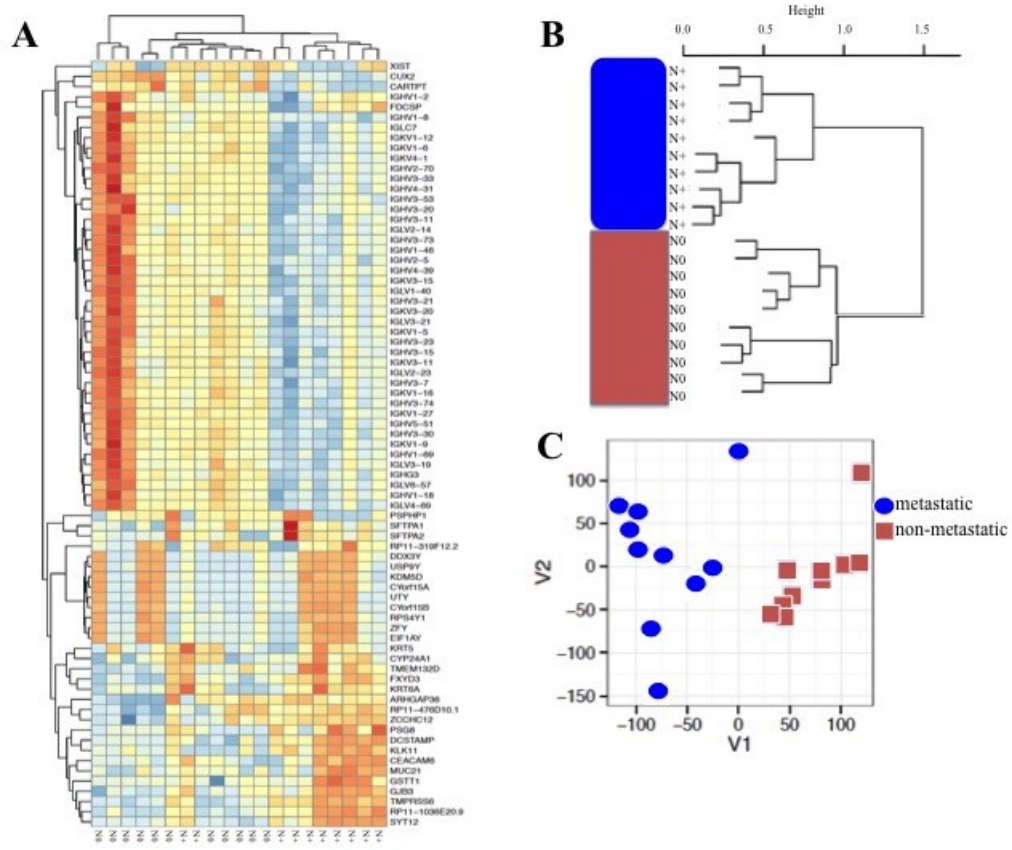
6.4. Results

RNA Seq shows high quality and ability to distinguish sample type

We used several metrics and exploratory analysis for data quality control and verification of biological data reliability. Pairwise sample correlation analysis to control the general transcripts expression consistency between samples was performed (Suppl. Table 4 (Appendix)). Samples belonging to the same design group are expected – and observed – to show higher level of correlation. Saturation plots were also constructed independently and for four different set of transcripts (high, intermediate, moderate, and low expressed transcripts (corresponding to quartiles Q1 to Q4 of median RPKM)) for each sample to show if there was enough sequencing depth to saturate gene expression. We calculated the Percent Relative Error (PRE), which measures how the RPKM estimated from a subset of reads deviates from real expression levels. One example of saturation plot is shown for sample 1N0 in Suppl. Fig. 3. The saturation plots for the remaining 19 samples are available upon request.

Using the top75 genes with maximal RPKM standard deviations, we identified the genes capable of differentiating samples from each other (Fig. 6.2 Panel A). Unsupervised hierarchical clustering on all genes was also conducted and confirmed the good quality of the isolated RNA, as it was capable of differentiating all but one metastatic samples from the others (Fig. 6.2, Panel B). Analogically, panel C shows multidimensional scaling (MDS) done on all metastatic and non-metastatic samples, showing good separations of clinical samples. Further, principal component analysis (PCA) also demonstrated a good separation of samples (Suppl. Fig. 4). Importantly, many of the top genes identified above are already known as "important diagnostic biomarker" for thyroid cancer, as recently reported in a recent meta-analysis of microarray based expression profile studies in thyroid cancer [121].

Figure 6.2: (A): Clustered heat maps of top 75 genes with the largest log2(RPKM) standard deviations. (B) Unsupervised hierarchical clustering on all genes. With the exception of one sample, all metastatic tumor cluster together. (C): Exploratory multidimensional scaling (MDS) done on metastatic and non-metastatic samples (Log2 CPM, baselined).



Differential gene expression analysis by RNA Seq shows consistency with qPCR validation

Differential gene expression analysis (DGEA) revealed 1008 protein coding genes (of 17380) to be differentially expressed between metastatic and non-metastatic samples (<5% FDR, P<0.01, log2FC>|2|) (Suppl. Table 5 (Appendix); Top 50 Heatmap, Fig. 6.3, Panel A). To validate RNA Seq based-DGEA, we performed qPCR on a series of genes (Fig. 6.3, Panel B) and compared the relative FC and p-value with the RNA Seq based results (Table 6.2). The relative FC between RNA Seq and qPCR-based experiments showed a correlation of 0.943 (Spearman's rho, P=0.005), thus showing high reproducibility of RNA Seq based DGEA.

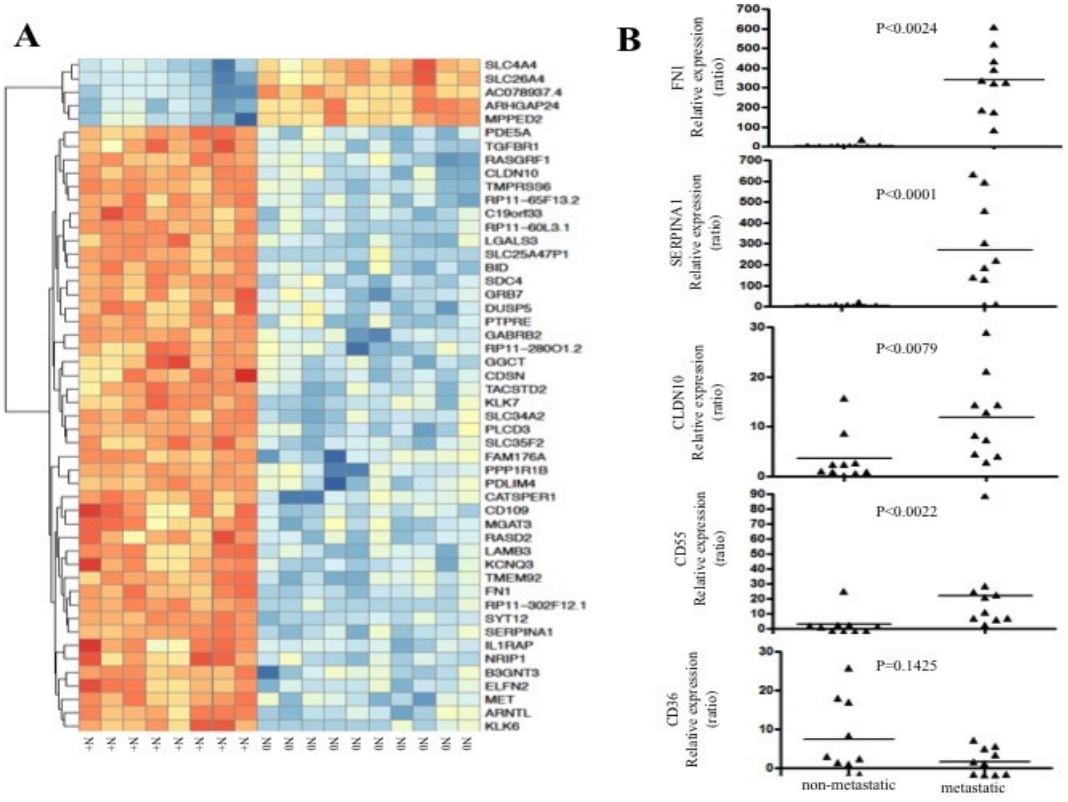
Among the overexpressed genes in metastatic samples, DAVID pathway analysis revealed cell surface markers to be highly commonly affected [122]. We found that 8.5% (83) of the affected genes are implied in cell adhesion, while 7.5% (73) are classified as cell-cell signalling genes. Moreover, 5.1% (50) were cell motion genes while 6.5% (63) of genes were related to immune

response. Among them, CD55 was recently reported to promote tumor escape from complement-mediated cytotoxicity [123]. CD36, also known as thrombospondin receptor, was shown to be a regulator of tumor infiltration and angiogenesis in PTC [124]. Further, serpins (e.g. *SERPINA1*) were recently reported to promote metastatic niche and protect invasive cells against host defense [125].

Table 6.2: Relative fold change and P value in DGEA between RNA Seq and qPCR

	RNA Seq		**qPCR**	
Gene	***FC***	***P value***	***FC***	***P value***
FN1	75.2	9E-28	63.03	<0.0001
SERPINA1	33.4	2E-19	79.75	0.0024
CLDN10	18.5	6E-19	12.09	0.0079
CD36	-2.8	0.002	-2.04	0.1425
CD55	7.05	5E-11	7.52	0.0022

Figure 6.3: (A): Clustered heat map showing top 50 genes differentially expressed between metastatic and non-metastatic samples. (B) Validation of RNA Seq by qPCR. Relative fold expression of selected genes between metastatic and non-metastatic samples.



BRAF mutation is associated with increase WNT pathway activation

Using Integrative Genome Viewer (IGV) [126], we screened for common mutations described in thyroid cancer [109] and compared how those impacted on RPKM expression of several genes. Among our 20 samples, 9 harboured an A to T substitution at position 140,453,136 on chromosome 7, that is harbouring a BRAF V600E classical mutation (Fig. 6.5). Interestingly, all of BRAF mutated samples but none of the BRAF wild-type (wt), showed overexpression of RSPO4 (FPKM>2 for BRAF V600E while FPKM<1 for BRAF wt) (Fig 6.5 Panel A). Overall, the relative fold-change between BRAF V600E and BRAF wt was about 10 and statistically highly significant (*P*=6.27E-07). First, we confirmed this result using qPCR and found similar FC using our own design primers. These primers were previously validated using a commercial RSPO4 plasmid kit (Suppl. Fig 5). Furthermore, an independent study by Smallridge *et al.* [127] comparing BRAF V600E to BRAF wt papillary thyroid cancers found similar expression patterns (Table 6.3). This suggests that BRAF V600E mutation results in activation of RSPO4 mRNA transcription.

To support this hypothesis, we knocked out the BRAF gene using CRISPR technology [128]. We found that both expression of RSPO4 protein and mRNA was decreased after BRAF knockout when compared to wt (Figure 6.5, Panel C and D). The antibody used to detect RSPO4 was also previously validated using a commercial plasmid overexpression kit (Suppl. Fig 5).

To further elucidate if RSPO4 was indeed capable of inducing WNT, we transfected two thyroid cancer cell lines (TPC1 and BCPAP)[118] with RSPO4 plasmid and analysed total cell extract, nuclear and cytoplasmic fraction of b-catenin. While transfection with RSPO4 resulted in modest increase of total and p-b-catenin (Figure 6.5E), it induced a dramatic increase in nuclear fraction of b-catenin, thus providing evidence of activation of the WNT pathway.

Table 6.3: Relative fold expression of RSPO4 in BRAF V600E mutant vs. BRAF wt.

	RNA Seq (this study)		**qPCR (this study)**		**RNA Seq (Smallridge *et al.*, JCEM, 2014)**	
Gene	***FC***	***P value***	***FC***	***P value***	***FC***	***P value***
RSPO4	9.82	6.27E-07	11.56	0.005	4.11	4.71E-05

Figure 6.4: BRAF mutation in RNA Seq. On the left is one example of mutated sample, showing A to T substitution, resulting in V to E amino-acid change at the position 600 of the BRAF kinase. On the right is an example of non-mutated sample.

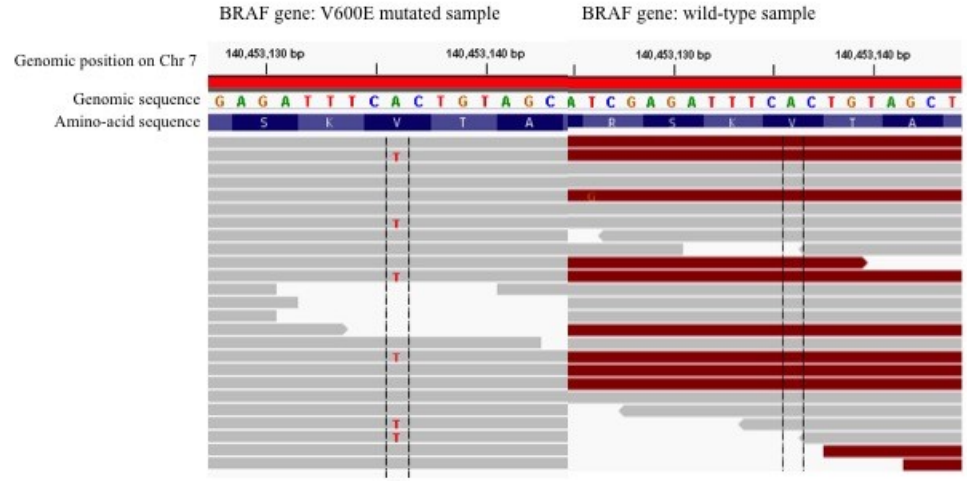
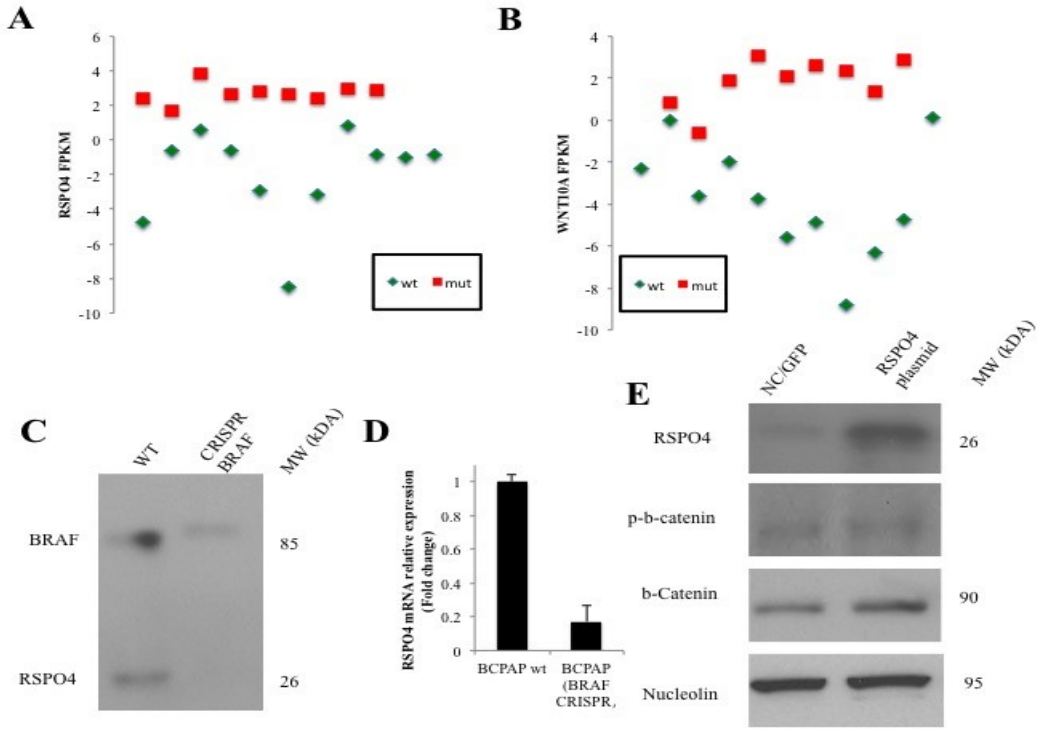


Figure 6.5: Expression of WNT related pathway factors in BRAF mutated samples (A) for RSPO4 and (B) for WNT10A. (C)&(D): BRAF and RSPO4 expression in BCPAP wt cells and CRISPR BRAF cells. The BRAF knockout resulted in under-expression of RSPO4 in both western blot (C) and qPCR analysis (D). (E): Transfection of BCPAP cells with RSPO4 plasmid, resulting in increase of RSP04 protein expression, as well as increase in beta-catenin levels. indicating activation of WNT pathway.



6.5. Discussion

In the current study we performed RNA Seq of 20 papillary thyroid carcinomas matched for age, sex and T category. Half of these samples were from cases with metastatic local lymph nodes, while the other half were metastasis free. The principal goal of this study was to identify in a wide-scale experiment specific expression features associated with metastasis in papillary thyroid cancer.

Importantly, we performed thorough quality controls, metrics and exploratory analyses, and validation by standard methods such as qPCR in order to assure the reproducibility and biological accuracy of our RNA Seq experiment. Although great amounts of data can be quickly generated by massive throughput technologies and can provide unprecedented integrative and comprehensive characterization of different biological conditions, one caveat is that it may be hard to identify a mistake, once massive parallel sequencing and bioinformatics pipelines are launched.

As the vast majority of papillary thyroid cancer shows an indolent behaviour, presenting clinically with negative nodes, biomarkers identified in this study will help characterize PTC thereby allowing for prediction of more aggressive phenotypes. This ability to predict tumor behaviour will enable clinicians to determine who require central neck dissection more or more complete thyroidectomy or postoperative RAI. The value and risks of prophylactic central neck dissection in papillary thyroid cancer remains a heavily debated subject. Central neck dissection bears increased risk of surgical complications while being of doubtful benefit [90, 92-95, 97]. Most molecular profiling studies have so far focused on increasing the diagnostic accuracy of fine needle aspirate biopsies (FNAB) [54, 129]. However, molecular profiling of metastatic thyroid tumors such as performed in this study could help out clinicians in planning the extent of their surgery preoperatively.

We identified over one thousand genes associated with metastasis in papillary thyroid cancer, among which many are linked to cell-adhesion, cell-cell signalling and immune status. This confirms the importance of tumor microenvironment and modulation of tumor microenvironment by tumor cells in the metastatic process [67, 130]. Recent major works have identified the

importance of the interaction between stromal cells and invasive tumor cells, promoting the integration of an immune function score into the AJCC/UICC classical TNM [131].

In the final part of the study, we focused our attention on a subgroup of papillary thyroid tumor harbouring a BRAF V600E mutation[46, 48, 110-112]. Interestingly, we found that all BRAF mutant tumors overexpressed members of the WNT pathway, such as *RSPO4* and *WNT10A* genes [132, 133]. Previous reports have suggested a crosstalk between BRAF and WNT pathway in colon cancer and melanoma, thus prompting us to further investigate this interaction in thyroid cancer [134-136]. We described for the first time in thyroid cancer, using CRISPR technology, an interaction between the BRAF/MAPK and WNT/b-catenin pathway. This finding is important as it may further explain the more aggressive phenotype seen in BRAF mutant thyroid tumors, but also, as RSPO4 is partially a secreted protein, may provide the opportunity to evaluate in further studies the utility of RSPO4 as a blood test in papillary thyroid cancer patients[133]. RSPO4, a member of the R-spondin family, was first described in congenital anonychia [137] and is a regulator of WNT pathway [138]. It was shown to promote stabilisation of b-catenin, thus promoting transition to mesenchymal state. Unlike thyroglobulin, RSPO4 seems to be tumor and BRAF V600E specific, thus eliminating diagnostic uncertainty due to physiological production of thyroglobulin in normal thyroid tissue.

Our first study provides the opportunity for further investigations. First, we constructed a thyroid tissue microarray with over 150 papillary thyroid cancer patients with long-term follow-up to investigate the potential diagnostic utility and predictive value of R-spondin in a clinical setting and at the protein level. Our data, alone or combined with other database, will expand the molecular understanding of thyroid cancer, necessary to be able to provide a tailored clinical approach.

In conclusion, we documented using "controlled" deep sequencing technologies, over a thousand genes overexpressed in metastatic papillary thyroid cancer, and characterize a novel function of RSPO4 - WNT pathway regulator – in a subset of BRAF mutant cancer.

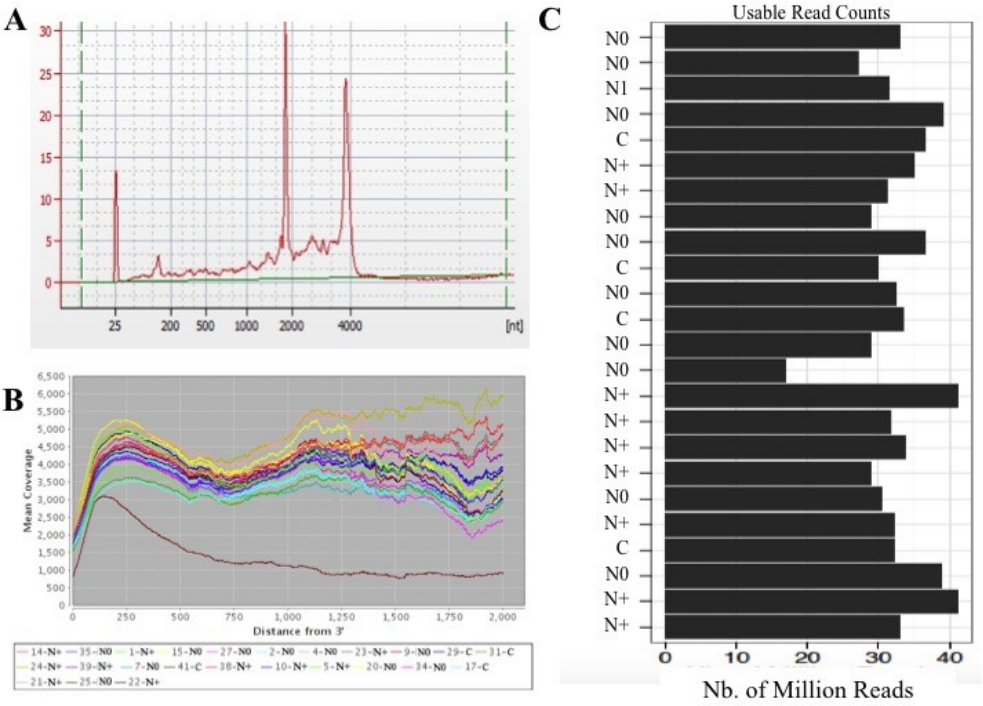
6.6. Acknowledgements

Supported by the Canadian Institutes of Health Research and in part by McGill University ENT Foundation. GBM is supported by the Swiss Cancer League, Effingerstrasse 40, 3001 Bern, Switzerland (BIL KFS-3002-08-2012).

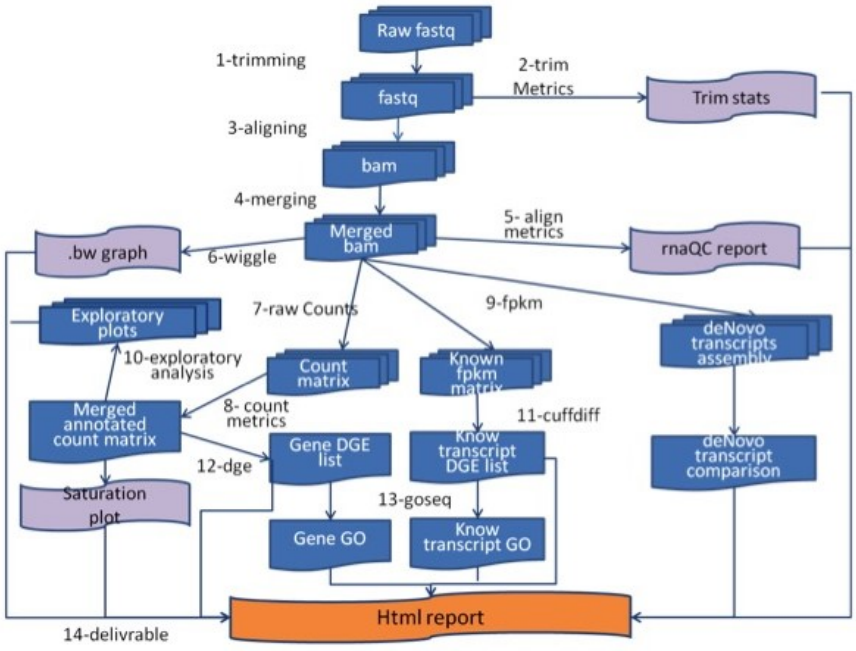
6.7. Supplemental Figures and Tables

Suppl. Figure 1: (A): Bioanalyzer graph shows high quality RNA with expected peaks at around 2000 and 4000 nt, representing 18S and 28S subunit of rRNA, respectively. All samples submitted for sequencing underwent bioanalyzer quality control before further downstream applications.

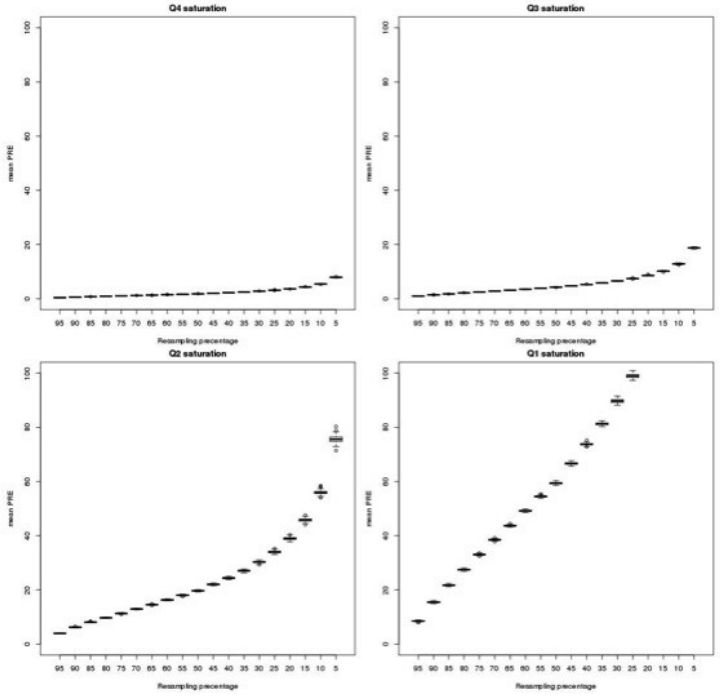
(B): Mean coverage from 3' during RNA Seq. (C): RNA Seq coverage for each sample.



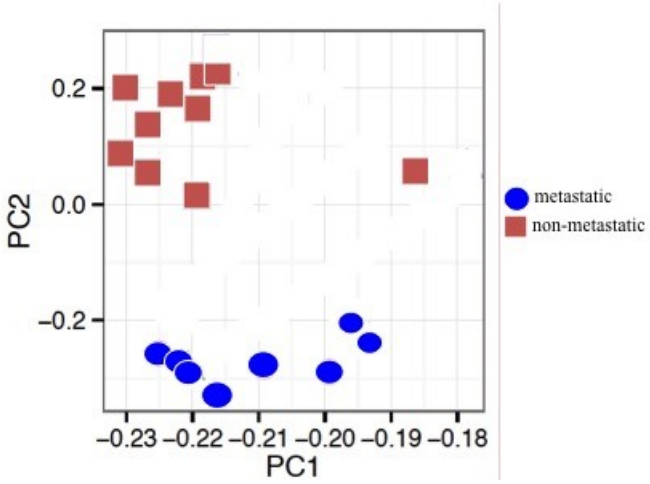
Suppl. Fig. 2: Internally developed RNA-Seq analytical pipeline at McGill University.



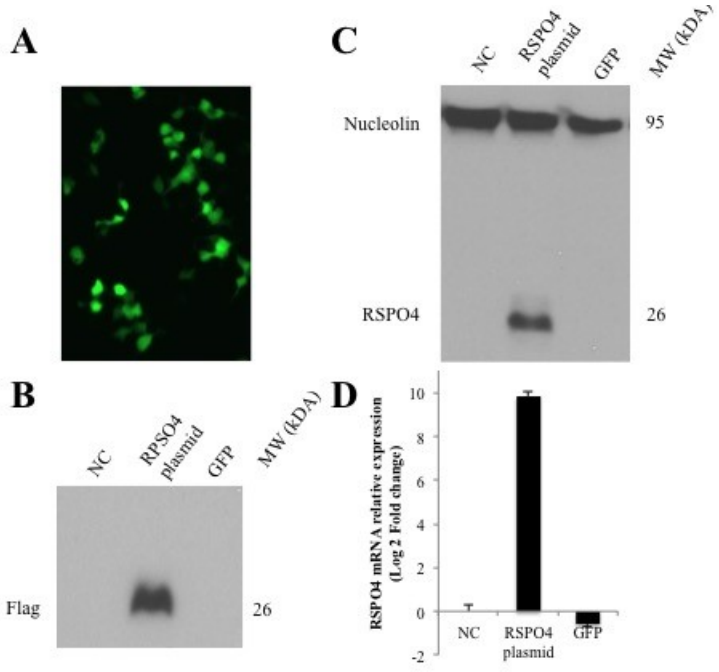
Suppl. Fig. 3: Saturation plot for sample 1N0 showing the Percent Relative Error (PRE) for each quartile Q1 to Q4 of median Reads Per Fragments Per Kilobase of exon per Million fragments mapped (RPKM). PRE measures how the RPKM estimated from a subset of reads deviates from real expression levels.



Suppl. Fig. 4: Principal component analysis (PCA) done on metastatic and non-metastatic samples, showing good separation



Suppl. Fig. 5: Validation of RSPO4 antibody and primers (A): Transfection control using GFP in HEK293 cells. (B) and (C): Western blot analysis of transfected HEK293 cells. A Flag-marked RSPO4 plasmid was used. Panel B shows blotting for Flag, while Panel C shows blotting for RSPO4 and loading control. (D): relative expression of RSPO4 mRNA in transfected cells, negative control (NC) and GFP transfected cells.



Supplemental tables: see appendix.

7. Manuscript 2: Insights into genetic and epigenetic determinants with impact on vitamin D signalling and cancer association studies: The case of thyroid cancer

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Running title: Genetic variations and vitamin D signalling in thyroid cancer.

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7.1. Abstract

Vitamin D is a key regulator of calcium metabolism and has been implicated as a cancer preventive agent. However, clinical studies have revealed conflicting results on its cancer preventive properties, attributed in part to multiple metabolic and regulatory factors susceptible to affect individual responses to exogenous vitamin D. Vitamin D is obtained from dietary sources and sun exposure, which depends on numerous parameters such as skin type, latitude, and lifestyle factors. Focusing on thyroid cancer, we document that genetic and epigenetic determinants can greatly impact individual response to vitamin D and may outweigh the classical clinical correlative studies that focus on sun exposure/dietary intake factors. In particular, genetic determinants innate to host intrinsic metabolic pathways such as highly polymorphic cytochromes P450s responsible for the metabolic activation of vitamin D are expressed in many organs, including the thyroid gland and can impact vitamin D interaction with its nuclear receptor (VDR) in thyroid tissue. Moreover, downstream regulatory pathways in vitamin D signalling as well as VDR are also subject to wide genetic variability among human populations as shown by genome-wide studies. These genetic variations in multiple components of vitamin D pathways are critical determinants for the re-valuation of the potential preventive and anticancer properties of vitamin D in thyroid cancer.

Keywords: Thyroid cancer, vitamin D, VDR, genome-wide studies, cancer susceptibility

7.2. Introduction

Thyroid cancer (TC) is the most common endocrine cancer malignancy worldwide [139] with a rising incidence in particular among young patients and women [3, 12, 13]. Overdiagnosis of subclinical disease, previously proposed as a contributor for the rising incidence, cannot explain the full extent of the increase [25, 26]. Risk factors such as exposure to ionizing radiation [19-22], chemical genotoxins [27-29], and obesity [30-33], as well as a lack of protective factors, such as vitamin D deficiency have been implicated in TC increased incidence [37-40].

Vitamin D, an active ingredient of cod-liver oil, was first identified as a cure for rickets in the 19th century and has emerged as a principal regulator of calcium homeostasis [140]. Cutaneous exposure to sun and dietary intake are the two main natural sources of vitamin D. Vitamin D activity depends on metabolic activation through hydroxylation of the 25 followed by the 1 position of the molecule by cytochromes P450s, which generate the biologically active metabolite 1,25$(OH)_2$D3. The action of vitamin D occurs mainly through its binding to the nuclear vitamin D receptor (VDR), which acts as a hormone-regulated transcription factor [141]. Upon activation, the VDR forms a heterodimer with related retinoid-X receptors and binds to vitamin D response elements (VDREs) on chromatin regions resulting in the regulation of the expression of several target genes [142-144]. VDRE binding by the VDR provides the principle mechanism by which the receptor can activate gene transcription. However, the hormone-bound receptor can also repress gene transcription by a variety of mechanisms [145]. Downstream targets of the receptor are involved in mineral metabolism, but VDR also regulates a variety of other metabolic pathways, many of which are components of immune response and cancer signalling [146, 147].

Independent studies support that circulating levels of vitamin D are inversely correlated to several malignancies, including colorectal cancer [148, 149], prostate cancer [150], breast cancer [151, 152], and head and neck squamous cell carcinoma [153, 154]. As well, a more recent meta-analysis reported a correlation between vitamin D deficiency and poorer prognosis in several tumor types [155]. In TC, several studies point towards a role for impaired 1,25$(OH)_2$D3-VDR signalling in the occurrence and progression of the disease [156]. This review addresses new insights into genetic and epigenetic determinants of vitamin D response in relation to cancer risk focusing on thyroid cancer. We provide a systematic review and analysis of experimental and clinical data and the impact of genome-wide analyses on individual susceptibility to TC.

7.3. Materials And Methods

Genomic database

The UCSC Cancer Genomics Browser [157], a set of web-based tools to display, was used to investigate and analyze cancer genomics data and its clinical information associated with VDR. The browser provides whole-genome to base-pair level views of several different types of genomic data, including next-generation sequencing platforms. Biological pathways, collections of genes, genomic or clinical information were used to sort, aggregate and zoom into a group of samples. The current release (2013) displays an expanding set of data from various sources, including 201 datasets from 22 TCGA (The Cancer Genome Atlas) cancers as well as data from Cancer Cell Line Encyclopaedia and Stand Up To Cancer [157].

Database of somatic mutations

To collect data on TC related to VDR mutation, the web-software BioMart Central Portal and the COSMIC (Catalogue of Somatic Mutations in Cancer) database [158] were used. BioMart offers a one-stop shop solution to access a wide array of biological databases, such as the major biomolecular sequence, pathway and annotation databases such as Ensembl, Uniprot, Reactome, HGNC, Wormbase and PRIDE [159]. The Cancer BioMart web-interface with the following criteria was used: 1. Primary site = “thyroid”; 2. Mutation ID is not empty. The first criterion ensures that the mutation occurs in thyroid tissues, and the second criterion helps to exclude the samples without mutation in a specific gene. Thereby we obtained the list of mutations in TC.

COSMIC [158] stores and displays somatic mutation information and related details in human cancer. COSMIC was developed, and is currently maintained, at the Welcome Trust Sanger Institute. It is designed to gather, curate, and organize information on somatic mutations in cancer and to make it freely available on-line. It combines cancer mutation data, manually curate from the scientific literature, with the output from the Cancer Genome Project (CGP). Genes are selected for full literature curation using the Cancer Gene Census. The current release (v64) describes over 913,166 coding mutations of 24,394 genes from almost 847,698 tumor samples. All genes selected for the COSMIC database came from studies in the literature and are somatically mutated in human cancer [160]. Based on this authority resource, a dataset of TC mutation was constructed.

Data extraction

Information was carefully extracted from all eligible publications including clinical and experimental studies assessing any relation between vitamin D and non-medullary TC. A search for studies in the electronic databases OvidMedline, OvidEmbase, Web of Science, AMED and the Cochrane Library was run using an elaborated search strategy (Supplemental material). In order not to miss any appropriate study, no time or language limits were applied for the search. Review articles were included only temporarily to provide a manual search tool.

The selection of studies involved an initial screening of the title and the abstract. In doubtful cases the full text was obtained. Articles were entered in the data management software and the duplicates were eliminated (Endnote 6®, Thompson Reuters Inc.). For clinical studies, detailed information about participants (number of patients, study location(s), demographics variables), exposure (sun irradiation, dietary intake, vitamin D serum level), comparison group and outcome was assessed.

The search retrieved 471 references published until July 4th, 2013, 12 from the Cochrane Library, 176 from OvidMedline, 188 from OvidEmbase and AMED, and 95 from Web of Sciences. Crosschecking the references of the reviews led to the inclusion of four supplementary articles [161-164]. No clinical trial was available. The flow chart of study selection is shown in Figure 7.1.

Overall 30 articles were included, of which 17 were clinical studies (Table 7.1) and 13 experimental studies (Table 7.2). These studies were published in English language from 1987 to 2013. Of the 17 clinical studies, eight (47.0%) showed protective effect of vitamin D [162, 163, 165-170], six (35.3%) no significant relationship [161, 164, 171-175] and two (11.7%) revealed an increased TC risk with high vitamin D intake [176, 177]. No comparison could be drawn from the remaining study (5.8%) [178]. TC incidence was assessed in all of these studies, mortality in two [163, 165]; and one report assessed both [163]. Except for three studies involving Arab populations [169, 174, 178], all studies included Europeans' descendants and/or Hispanic whites.

Figure 7.1: Flow chart of study selection for systematic review.

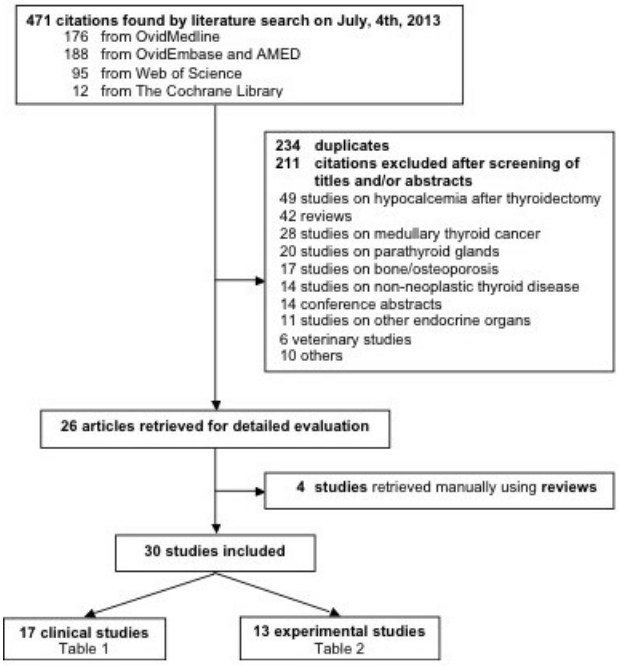


Table 7.1: Summary of clinical studies reporting an association between thyroid cancer and vitamin D.

First author	Pub year	Country (State/Province)*	Cases/ controls	Outcome	Exposure	Results[$]
Akslen [162]	1998	Norway	2627/NA	Incidence	Seasonal variation	pro
Boscoe [163]	2006	USA	>4'000/ >4'000	Incidence & Mortality	Latitude	pro[§]
D'avanzo [171]	1997	Italy	399/617	Incidence	Intake	NS
Glattre [172]	1993	Norway	92/460	Incidence	Intake	NS
Grant [165]	2006	Spain	NR	Mortality	Latitude	pro
Greenlee [176]	2004	USA (WA)	305/ 64'226	Incidence	Intake	con
Laney [173]	2010	USA (NE)	24/42	Incidence	Serum 25(OH)D	NS
Mack [161]	2002	USA (CA)	292/292	Incidence	Intake	NS
Penna-Martinez [166]	2009	Germany	147/57	Incidence	Serum $1,25(OH)_2$ D	pro
Penna-Martinez [167]	2012	Germany	253/302	Incidence	Serum $1,25(OH)_2$ D	pro
Peterson [178]	2011	USA (MI)	30/70	NA	Sun exposure	NA
Ron [177]	1987	USA (CT)	159/285	Incidence	Intake	con
Roskies [168]	2012	Canada (QC)	12/88	Incidence	Serum 25(OH)D	pro
Sahin [169]	2013	Turkey	344/116	Incidence	Serum 25(OH)D	pro
Stepien [170]	2010	Poland	50/26	Incidence	Serum $1,25(OH)_2$ D	pro

** WA: Washington, NE: Nebraska, CA: California, MI: Michigan, CT: Connecticut, QC: Quebec; $: pro: protective effect of vitamin D (or surrogates). NS: not significant. con: vitamin D (or surrogates) increasing risk. NA: not applicable. § for women only*

Table 7.2: Summary of experimental studies using cell lines or preclinical models to assess vitamin D effect on thyroid cancer.

First author	Pub year	Samples[§]	Main results
Balla [191]	2011	6 PTC	Overexpression of CYP24A1 mRNA
Bennett [190]	2012	TPC1, C643	Antiproliferative effect of calcitriol
		TPC1, KTC, BCPAP, FTC133, C643, Hth7	CYP24A1 downregulation
Brown [216]	2006	5 PTC	Vitamin D binding protein overexpression
Clinckspoor [209]	2011	FTC133, C643, 8505c, Hth74	Antiproliferative effect of calcitriol and superagonistic analogue CD578
Clinckspoor [192]	2012	64 thyroid cancers	VDR, CYP24A1, CYP27B1 overexpression
Dackiw [213]	2004	15 SCID mice/WRO	Growth inhibition of orthotopic tumour and p27 restoration after calcitriol treatment
Haghpanah [174]	2007	71 DTC	No difference in VDR polymorphism compared to healthy patients
Khadzkou [193]	2006	44 PTC	Overexpression of VDR and CYP27B1 (FFPE)
Liu [210]	2002	NPA, WRO	Antiproliferative effect of calcitriol and superagonistic analogue EB1089, p27 restoration
Liu [214]	2005	WRO	Calcitriol and EB1089 restore PTEN-dependent fibronectin expression
		SCID mice/WRO	Growth inhibition in heterotopic model with calcitriol and EB1089
Liu [215]	2011	WRO, MRO	Calcitriol inhibits CEACAM1
Okano [211]	1999	Nude mice/NPA	Trend to growth inhibition in heterotopic model with calcitriol and less-calcemic analogue
		NPA	Dose-dependent inhibition of calcitriol and less-calcemic analogue
Sharma [195]	2010	TPC1, C643, Hth7, Hth74, 8505c, SW1736	Differential VDR expression
			Cell cycle inhibition with calcitriol only in TPC1
Somjen [194]	2013	NPA, ARO, MRO	Overexpression of VDR and CYP27B1
Suzuki [212]	1999	TPC1-4	Dose-dependent growth inhibition of calcitriol and less-calcemic analogue
		TAC1, TTA1	
Wang [217]	2001	FRO, KAT5, NPA, WRO	Bcl2 upregulation with calcitriol

§: Cell line-corresponding histologic subtype: TPC1-4-PTC, KTC-PTC, BCPAP-PTC, NPA-PTC, KAT5-PTC, FTC133-FTC, FRO-FTC, MRO-FTC, WRO-FTC, C643-ATC, Hth7-ATC, Hth74-ATC, 8505c-ATC, SW1736-ATC, TAC-1-ATC, TTA-1-ATC. PTC= papillary thyroid cancer, FTC: follicular thyroid cancer, ATC: anaplastic thyroid cancer. SCID=severe combined immunodeficient.

7.4. Results And Discussion

Determinants of vitamin D levels and impact in TC

Solar UVB irradiation is the primary source of vitamin D and can be estimated by latitude of the living area. In TC, large epidemiological studies support an inverse relation between TC incidence and latitude [163, 165] (Table 7.1). These studies performed a multivariate analysis to adjust for confounding factors. However vitamin D levels were not measured. Consequently, it is unclear if the multivariate analysis resulted in accurate vitamin D estimates. Indeed, vitamin D deficiency is highly prevalent among latitudes that benefit from high solar irradiation such as Africa, the Middle East, and Southern Asia. This may be due to skin pigmentation, traditional clothing, and sun avoidance seen in southern heat-exposed populations [178, 179]. In contrast, fair-skinned northern populations usually seek sun exposure and may also benefit from high intake of vitamin D rich diet such as fatty fish and cod liver oil [179]. Further, a mutation in the cutaneous structural protein fillagrin, which occurs in up to 10% of Europeans was shown to lead to higher circulating vitamin D levels [180]. Nonetheless, North American and European studies have shown seasonal variations of vitamin D levels due to insufficient sun irradiation during winter [181]. In TC, one study from Norway reported higher proliferation values for tumors resected during winter compared to other seasons [162]. These results comply with above-mentioned studies showing an inverse relation between TC incidence and latitude [163, 165]. For studies estimating vitamin D consumption and TC risk, however, no convincing associations have been shown (Table 7.1) [161, 171, 172, 176, 177]. This may be due to the general poor correlation between vitamin D deficiency and estimates of vitamin D consumption [175].

A more accurate way to assess vitamin D is biological monitoring. Association studies investigating the relationship between levels of serum vitamin D and TC risk mostly point towards a protective effect of vitamin D [166-170, 173, 182] (Table 7.1). Pooling the data among these studies is not possible due to different cut-off levels for different vitamin D derivatives and control groups used in each of these studies. This would greatly limit the validity of a meta-analysis. The lack of consensus in cut-off levels may reflect the fact that those are differently defined depending on targeted clinical endpoints [183, 184]. Classical vitamin D targets, i.e. those implicated in calcium and bone homeostasis, do not allow conclusions on optimal level of vitamin D having anticancer properties. While doses up to 4,000 IU of daily vitamin D supplementation have been considered safe, studies have reported hypercalcemia, nephrolithiasis, vascular and soft tissue calcification with high doses of vitamin D and also U-shaped relationship

between vitamin D levels above 75nmol/l and certain cancer subtypes [185, 186]. One additional issue of most of these association studies is that vitamin D levels were measured only once, which does not permit distinction between outcome and exposure. Indeed, some studies have reported low serum vitamin D as a result of malignancy [187].

Above mentioned skin types, alimentary, and social habits yet do not fully explain vitamin D variability among populations [188]. One major determinant of individual susceptibility to vitamin D is the activity of vitamin D metabolizing enzymes. Three major cytochrome P-450 (CYP) hydroxylases are responsible for vitamin D activation through 25- followed by 1α-hydroxylation of the molecule, and deactivation through 24-hydroxylation. Multiple enzymes have been reported as vitamin D 25-hydroxylases, a step occurring constitutively and primarily in the liver. Unlike 25-hydroxylation, 1α-hydroxylation of $25(OH)D_3$ by the CYP27B1 is a tightly regulated and rate-limiting step. It is regulated by calcium, $1\alpha,25(OH)_2D_3$ itself, PTH, calcitonin, and phosphate levels. Recently, fibroblast growth factor 23 (FGF23) was identified as a novel antagonist of PTH and is thought to play an important role in vitamin D regulation pathway [189]. Although CYP27B1 and CYP24A1 are primarily expressed in the kidney, recent studies showed that they are expressed in many other tissues, including the thyroid [190, 191]. In TC, there is evidence that polymorphisms leading to impaired CYP27B1 function and/or increased CYP24A1 activity are associated with increased TC risk [167]. Transcriptional profiling studies show that both enzymes are overexpressed in early TC [191], but their expression tends to decrease along with tumor progression [192, 193].

Determinants of predicted response to vitamin D

The action of vitamin D mainly occurs through binding to the VDR [141], whose levels are subject to genetic variations. Using the UCSC genomic database, we analyzed 552 thyroid samples that underwent genomic profiling using RNA Seq. The expression of VDR was down regulated in benign thyroid samples and up regulated in most TC cases (Figure 7.2). These results are confirmed by a few *in vitro* studies using thyroid cancer cell lines [194] and independent clinical samples [192, 193]. However, VDR levels alone may translate poorly with response to vitamin D stimulation if polymorphisms of VDR are not taken into account [195-197]. The analysis of the genomic organisation of the VDR *locus* at chromosome 12q13.1 revealed the large *VDR* gene (about 100Kb) with an extensive promoter region capable of generating multiple tissue-specific transcripts [198]. In view of the observed genome-wide frequency of single

nucleotide polymorphisms [199], one can predict >100 functional polymorphisms to be present in the VDR region alone, including the promoter region (Figure 7.3). Point mutations in the VDR gene have been identified in various regions, including the VDR DNA binding domain (DBD) and the ligand binding domain (LBD) [200]. Such mutations can disrupt ligand-binding affinity to the receptor [201], heterodimerization of VDR with RXR [202], or interactions of the VDR receptor with partners such as coactivators [203]. Other mutations such as in the initiation codon can create a premature termination [204] or alternative translation start sites to result in alternative splicing and formation of truncated proteins [205, 206]. The analysis of the COSMIC database showed a high proportion of missense mutations that were re-identified (67.44%), while complex mutations were not detected (Table 7.3). The distribution of the mutations observed in the VDR gene in TC is shown in Figure 7.4. Only two studies investigated the association between VDR polymorphisms and TC risk, one showed an increased TC risk for patients with particular VDR polymorphism [166], while another could not point out any significant difference [174].

Table 7.3: Mutations identified in VDR**.**

Position (AA)	Mutation (CDS)	Mutation (Amino Acid)	Mutation Type
8	c.23C>T	p.T8I	Substitution - Missense
33	c.98G>A	p.G33D	Substitution - Missense
52	c.156G>A	p.M52I	Substitution - Missense
74	c.221G>A	p.R74H	Substitution - Missense
78	c.233C>G	p.A78G	Substitution - Missense
130	c.389G>A	p.R130H	Substitution - Missense
146	c.438C>G	p.T146T	Substitution - coding silent
149	c.445G>T	p.D149Y	Substitution - Missense
154	c.460C>T	p.R154W	Substitution - Missense
158	c.472C>T	p.R158C	Substitution - Missense
159	c.477G>C	p.V159V	Substitution - coding silent
161	c.481G>A	p.D161N	Substitution - Missense
162	c.484G>T	p.G162C	Substitution - Missense
169	c.507G>A	p.R169R	Substitution - coding silent
181	c.541G>T	p.D181Y	Substitution - Missense
191	c.573C>A	p.I191I	Substitution - coding silent
199	c.597G>A	p.S199S	Substitution - coding silent
208	c.623G>T	p.S208I	Substitution - Missense
236	c.708C>A	p.Y236*	Substitution - Nonsense
253	c.757G>T	p.D253Y	Substitution - Missense
274	c.820C>T	p.R274C	Substitution - Missense
296	c.887G>A	p.R296H	Substitution - Missense
320	c.960G>A	p.L320L	Substitution - coding silent
339	c.1015G>A	p.V339I	Substitution - Missense
350	c.1049C>T	p.A350V	Substitution - Missense
350	c.1050G>A	p.A350A	Substitution - coding silent
352	c.1056T>C	p.I352I	Substitution - coding silent
353	c.1058A>T	p.E353V	Substitution - Missense
358	c.1072C>T	p.R358C	Substitution - Missense
365	c.1094C>T	p.T365M	Substitution - Missense
368	c.1103G>A	p.R368H	Substitution - Missense
379	c.1135C>T	p.L379F	Substitution - Missense
399	c.1196A>T	p.K399M	Substitution - Missense
402	c.1205G>C	p.R402P	Substitution - Missense
418	c.1254G>T	p.V418V	Substitution - coding silent
420	c.1258G>A	p.E420K	Substitution - Missense
?	c.907+1G>T	p.?	Unknown

Figure 7.2: Gene expression profile showing VDR signature for 552 thyroid cancer cases (RNA Seq). Each row corresponds to sample from a single case. Columns from the left correspond to genomic heat map according to chromosomal location. The last two columns represent VDR expression profile (represented by red for overexpression and green for downregulation) in normal (pink) versus cancer (red) tissues. VDR is mostly overexpressed in malignant samples but almost absent in benign tissues. Source: UC Santa Cruz - Cancer Genomics Browser.

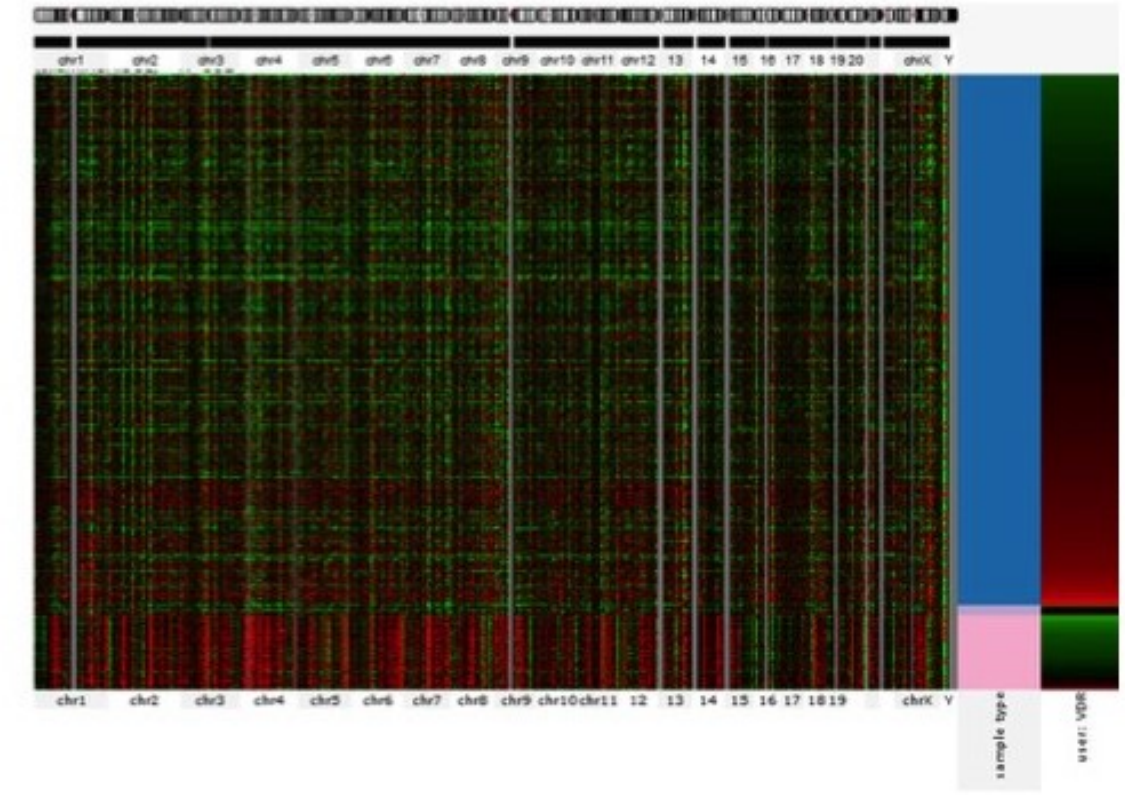


Figure 7.3: Schematic diagram of VDR gene showing different restriction site on chromosome 12.

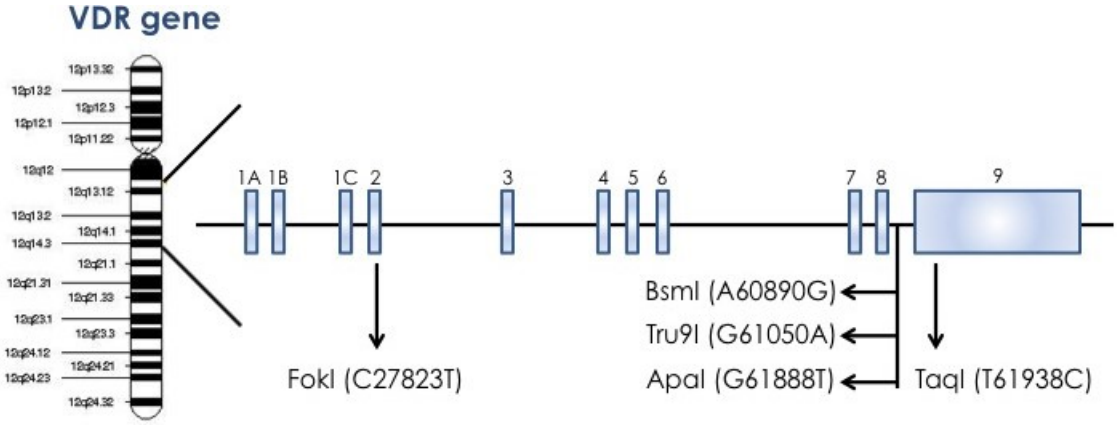
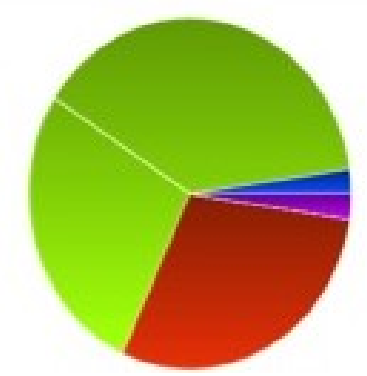


Figure 7.4: Pie-chart showing the percentage of the mutation type in VDR in thyroid cancer according to COSMIC database.

Color	Mutation Type	Mutant samples	Percentage
	Substitution nonsense	1	2.33
	Substitution missense	29	67.44
	Substitution synonymous	13	30.23
	Insertion inframe	0	0.00
	Insertion frameshift	0	0.00
	Deletion inframe	0	0.00
	Deletion frameshift	0	0.00
	Complex	0	0.00
	Other	1	2.33
	Total	43	100



Impact of VDR activation on intracellular signalling

Upon activation by vitamin D, VDR binds as a heterodimer with retinoid X receptors to specific VDREs [189]. VDREs usually bear a consensus sequence known as DR3 element located in the promoter region of the target genes. In addition to this classic mechanism, recent chromatin-immunoprecipitation (ChIP-seq) studies allowed to gain genome-wide insights of the binding sites of VDR [207]. These studies showed that the ligand-bound heterodimer can bind to ~2000-8000 sites in the genome. Interestingly, the majority of the binding sites do not bear the classical DR3-type sequence [189]. A significant enrichment was seen in regions associated with active chromatin and histone modifications thus supporting a broad genetic and epigenetic regulatory role of vitamin D. Further enrichment of VDR binding was also found in proximity of genes involved in autoimmune diseases (e.g. multiple sclerosis, type-I diabetes, Crohn's disease) and colorectal or breast cancer [208]. For TC, only data relying on classical *in vitro* experiments is available.

In agreement with experimental studies in other cancer types, exposure of a variety of TC cells to vitamin D *in vitro* leads to antiproliferative and pro-differentiation properties [190, 195, 209-212] (Table 7.2). These results have been confirmed by *in vivo* studies [213, 214]. Most studies are testing vitamin D itself and synthetic vitamin D analogues, as patient's exposure to pharmacologically high doses of vitamin D can be limited by the side effects, mainly hypercalcemia [195, 209-212].

Mechanistically, vitamin D was shown to inhibit proliferation through c-mac mRNA inhibition, which is a well-known proto-oncogene [211]. Further, it can induce a growth arrest effect in part through stimulating accumulation of the cyclin-dependent kinase inhibitor $p27^{kip1}$ in the nucleus [210]. Treatment with vitamin D is thought to prevent $p27^{kip1}$ phosphorylation, which was shown to increase its ubiquitin-dependent proteasome degradation [210]. Further, vitamin D was shown to enhance cell-cell adhesion through PTEN-dependent fibronectin upregulation [214]. Those results could be confirmed *in vivo*. Interestingly, the antiproliferative effect of vitamin D was abolished when knocking down fibronectin [214] and was shown to be independent of CEACAM1 expression, a tumor-suppressive adhesion molecule [215].

7.5. Conclusion and perspectives

The pleiotropic roles of vitamin D in cancer have been recognized through seminal preclinical studies although the preventive and therapeutic potential of vitamin D or its analogues remain debated due in part to the complex mode of action of this vitamin. Recent progress in high-throughput technologies to interrogate human genomic and epigenomic events has provided additional levels of regulatory loops and individual genetic variations that can impact on individual susceptibility to vitamin D. This knowledge opens up new tools to address confounding factors that contribute to discrepant results seen in previous association studies, in particular in relation to cancer prevention. As well, this knowledge impels an exciting avenue in the discovery of novel vitamin D analogues with enhanced preventive or therapeutic efficiency and limited side effects.

7.6. Acknowledgments

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8. Manuscript 3: *A*ntineoplastic activity of a novel purine-derived small molecule targeting poorly differentiated anaplastic thyroid cancer cells expressing elevated ALDH1 activity

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Study founding

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Abbreviated title: A novel purine derivative for anaplastic thyroid cancer

Mini Abstract (36 words, max 50 words).

In this experimental study, we report the antineoplastic activity of a novel purine derivative (SLL02) in anaplastic thyroid cancer. We show that SLL02 is capable of inhibiting multiple kinases and reprogramming of cancer stem cell-like markers.

Key terms: anaplastic thyroid cancer, purine derivative, Aurora, kinase, cancer stem cell

Word count: 2960/3500

Number of figures and tables: 7 (max 7).

Disclosure statement:

The authors have nothing to disclose.

8.1. Structured abstract

Objective: The objective of this study was to investigate the potential therapeutic benefit of a novel purine-derived (SLL02) multi-kinase inhibitor.

Background: Anaplastic thyroid carcinoma (ATC) is one of the most aggressive human malignancies characterized by de-differentiation features of malignant follicular thyroid cells. For ATC, no targeted therapy is available yet and conventional chemotherapy has only a marginal efficacy with high rates of relapses.

Methods: A novel purine-derivative (SLL02) was synthetized and evaluated for antineoplastic activity against ATC in preclinical models. Target kinase inhibition of SLL02 was screened by kinase assay and confirmed in vitro. Further, phenotypic changes induced by SLL02 were evaluated by FACS analysis.

Results: Exposure of the anaplastic thyroid cell line 8505c to the inhibitor at nM concentrations *in vitro* inhibited cell proliferation using the colony formation assay, as well as cell locomotion using the wound-healing assay. In a mouse model, treatment with SLL02 at doses of up to 30 mg per kg given as multiple cycles reduced significantly the growth of ATC tumors induced by heterotopic implantation of the 8505c thyroid cancer cells. Mechanistically, SLL02 was found to inhibit Aurora A, B and C, as well as RET and SRC kinases. This molecule was also found to induce cell differentiation reprogramming with reduction of ALDH+ cancer stem cell-like subpopulations and decreased *ALDH1A1* mRNA levels.

Conclusion: Our data support the potential clinical utility for the use of SLL02 in ATC.

8.2. Introduction

Thyroid cancer is the most common endocrine malignancy worldwide [1]. The vast majority of thyroid malignancies are well differentiated and long-term survival can be achieved by surgery and, if necessary, radioactive iodine. However, progressive dedifferentiation of thyroid cancer can give rise to anaplastic thyroid carcinoma (ATC), resulting in highly aggressive malignancy with fatal outcome [6-8]. Current treatment options in ATC show only marginal efficacy, confirming the urgent need for innovative therapeutic strategies in ATC [218].

ATC is thought to result from the activation of the MAPK and PI3K-AKT signalling pathways and cumulative mutations in oncogenes such as TP53, CTNNB1, BRAF and RAS [45, 48, 49, 51, 53]. While the accumulation of key mutations favours the concept of the classical multifactorial and multistage carcinogenic process [9], recent evidence supports a role of stem cell-like subpopulation in ATC [219-222]. These cells are not only self-renewing but are also both tumorigenic and believed to be intrinsically resistant to cytotoxic chemo- and radiation therapies [64-66].

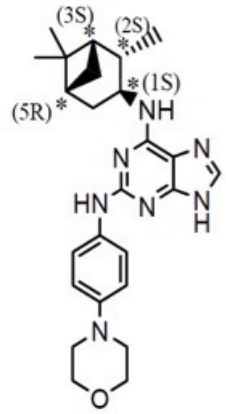
Highly aggressive tumors like ATC are characterized by high mitotic rate, aberrant activation of transcription and enhanced enzymatic activity of multiple kinases. Among them, the involvement of Aurora kinases in cancer development is of particular interest as it represents a potential therapeutic target in ATC for the inhibition by small molecules [223-225]. However, inhibition of a single pathway can be overcome by multiple resistance mechanisms, prompting the use of multi-targeted inhibitors or cocktails of selective antagonists [226]. Inhibition of ALDH was proven to decrease both cancer cell proliferation and motility[227].

In this study, we investigated the potential therapeutic benefit of a novel purine-derived (called SLL02) multi-kinase inhibitor. Our results demonstrate *in vitro* inhibition of multiple kinases. Also, we demonstrated that SLL02 is a potent inhibitor of ALDH-positive cancer stem cell-like subpopulation. Functionally, this translated into reduced cell motility and colony forming *in vitro*. These results were confirmed using an *in vivo* murine anaplastic thyroid cancer model.

8.3. Materials and Methods

Chemical structure and synthesis of SLL02: The figure and chemical formula of SLL02 is shown in figure 8.1. SLL02 was synthetized in our laboratory by DW, an experienced chemist.

Figure 8.1: Chemical structure of SLL02.



SLL02:
isopinocampheylamine-
substituted purine

Cell lines: 8505c and TPC1 cell lines are a courtesy of Dr. M. Trifiro (Department of Endocrinology, Jewish General Hospital, McGill University) and were purchased from DSMZ—German collection of microorganisms and cell culture [118]. 8505c is derived from a undifferentiated thyroid tumor of 78-year-old-female patient, and was maintained in culture in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum (Mediatech Inc.) and 50U/mL of penicillin-streptomycin (1%) [228].

Clonogenic assay: Clonogenic assays were performed using standard techniques as described elsewhere [229]. Briefly, cells were harvested from exponential-phase cultures, counted and plated at densities of 200 cells per well of a 12-well plate for 8505c cells (Corning® Costar®). Twenty-four hours after plating, the coumpoundss were delivered once at the mentioned concentration. Negative control was carried out by treating the cells with DMSO. After 6 days of incubation in the presence of the coumpounds, the cells were stained with 0.5% crystal violet in absolute ethanol, and colonies were counted using the machine GelCount™ Tumor Colony Counter from Oxford Optronix Ltd. Each experiments was done in triplicates and each clonogenic graph was constructed from at least three independent experiments using the average survival counts.

In vitro motility assay: The cell motility was investigated using the scratch motility assay as described earlier [230]. In this study, cells were grown on sterile coverslips for 24 h and were

then wounded by cell scraping using a micropipette tip. Following the culture washing, the cells were incubated at 37 °C in the presence of compounds for the reported time period to allow the migration toward the gaps, and the wound healing areas were examined under the microscope.

In vivo animal studies: Animal experiments were performed in compliance with institutional and federal guidelines after full approval from the McGill University Facility Animal Care Committee. (Protocol #5018). RAG mice obtained from Charles River Laboratories (St. Zotique, Quebec, Canada). Exponentially growing cells were suspended in PBS (0.5 x 10^6 cells per 10µl) and injected using a xenograft heterotopic model of 6-7 female RAG mice per group [231]. After cleaning of the surgical zone, 10 millions 8505c cells were injected subcutaneously. Thereafter, mice were assessed clinically everyday and weighted twice weekly day. According to safety standard operating procedure, animals loosing more than 20% of their initial weight or showing clinical signs of distress were sacrificed. Treatment was begun 15 days after injection of the tumor cells. Upon necropsy, tumor were dissected and weighted using a precision balance. The negative control was carried out by injecting vehicle alone, while the positive control was done using paclitaxel i.p. according to ATA Guidelines for anaplastic thyroid carcinoma [218, 232].

Kinase assay and Western blot analysis: SelectScreen ® kinase profiling on SLL02 was performed by Life Technologies. Screening results were then confirmed by Western blot analysis. Total cell extracts from exponentially growing cells were collected by scrapping into modified radioimmunoprecipitation assay lyses buffer supplemented with 20mg/mL pepstatin A, 1mM PMSF, and protease inhibitor cocktail (Roche). SDS-PAGE was performed with standard methods. Blots were detected using specifics antibodies in accordance with the manufacturer's recommendations and the signal was detected with peroxidase–conjugated secondary antibodies and enhanced chemiluminescence detection system. When indicated, membranes were subsequently stripped for reprobing with internal controls such as GAPDH [233].

FACS analysis and sorting: For FACS analysis of cell surface markers, live cells were collected after digestion with 0.25% trypsin. Cells were suspended in blocking buffer (1% BSA and 10% FBS in PBS with 0.01% sodium azide) for 30 minutes at room temperature to reduce non-specific binding. For direct FACS analysis of CD24 and CD44, 10^6 cells were co-stained with 5uL of alexa-fluor647-conjugated CD24 antibody (Biolegend) and 5uL FITC-conjugated CD44 antibody (Biolegend) in 100uL final volume of blocking buffer for 1 hour, followed by 3 PBS washes. For

indirect FACs analysis of CD133, cells were incubated with 1:1000 CD133 primary antibody (Miltenyi Biotec) for 1 hour, followed by 3 PBS washes, stained with 1:1000 alexa fluor 488-conjugated anti-mouse secondary (life technologies), followed by 3 more PBS washes. Primary and secondary staining was done at room temperature. For FACS experiments, non-viable cells were gated out by co-staining with 7-AAD. Non-specific isotype controls were used as negative control in direct FACS experiments, and a secondary-stained antibody was used as negative control in indirect FACS experiments. To quantify the percentage of positively stained cells, the gating was set such that the negative control group displayed less than 0.5% positive signals. At least 10,000 live cell events were collected for each sample. The samples were analyzed by flow cytometry using BD FACScalibur and the data was analyzed using FCS Express software. The enzymatic activity of ALDH was detected using ALDEFLUOR staining kit (StemCell Technologies). Cells were harvested after 72h treatment period during which the media and the coumpound were refreshed one. After trypsinisation and washing of the cells, each sample was re-suspended in ALDH assay buffer and split into 2 tubes, one with + DEAB and one with – DEAB. The DEAB acts as a negative control by inhibiting ALDH reporter activity. The cells were incubated in 100ul of assay buffer + 1ul of 300NM ALDEFLUOR reagent and 1ml of DEAB reagent for 40 minutes at room temperature. The cells were then centrifuged and re-suspended in new assay buffer + 5ul of 7-AAD. 7-AAD staining is used to exclude necrotic cells during FACS analysis. The ALDEFLUOR intensity is measured using BD FACScalibur using the FL-1 channel.

Real-time PCR: Fifty thousand 8505c cells were plated in 6-well Corning® Costar® plates. Two days after, they were treated with increasing concentration of SLL02. Treatment with DMSO was used as a negative control. Twenty-four hours after treatment, RNA was extracted using total RNA Mini Kit (Genaid ® FroggaBio ®) according to the manufacturers' instructions. cDNA was synthesized from 500 ng of RNA using Superscript II reverse transcriptase (Invitrogen®) and oligo-dT primers (Invitrogen®). qRT-PCR was performed in the ABI PrismTM 7900 (Applied) using SYBR® Green (Applied) in a 10 µL total volume and quality controls were used according to the MIQE Guidelines [119]. The reactions were carried out in triplicate. *HRTP1* was used as endogenous control. Following sequences were used: ALDH1A1: F-5' CCA GCC CAC AGT GTT CTC TA. R-5' TGT TTG CTC TTT TGA TCA CGT. ALDH1A3: F-5': ACC GAC TAT GGA CTC ACA GCA. R-5': GTT CTC TGC CAT TTC CTG ACA. ALDH2: F-5': ATG GCA

AGC CCT ATG TCA TCT. R-5': CCG TGG TAC TTA TCA GCC CA. Fold differences in the relative gene expression were calculated using Pfaffl model [120]

Statistical analysis: For dichotomous variables, the two-sided Fischer Exact test was used to compare proportions. Odds ratio (OR) and 95% confidence interval (95%CI) were calculated according to the Mantel-Haenzel method. For discrete variables showing normal distribution, means and standard errors of means (SEM) are given and comparisons were made using the t-test. Alternatively, median, first quartile (Q25), and third quartile (Q75) are indicated and the non-parametric Kruskal-Wallis test was used. Statistical analyses were performed using SPSS® 21.0.0 software (IBM©, Armonk, NY, US).

8.4. Results

SLL02 inhibits colony forming and cell motility

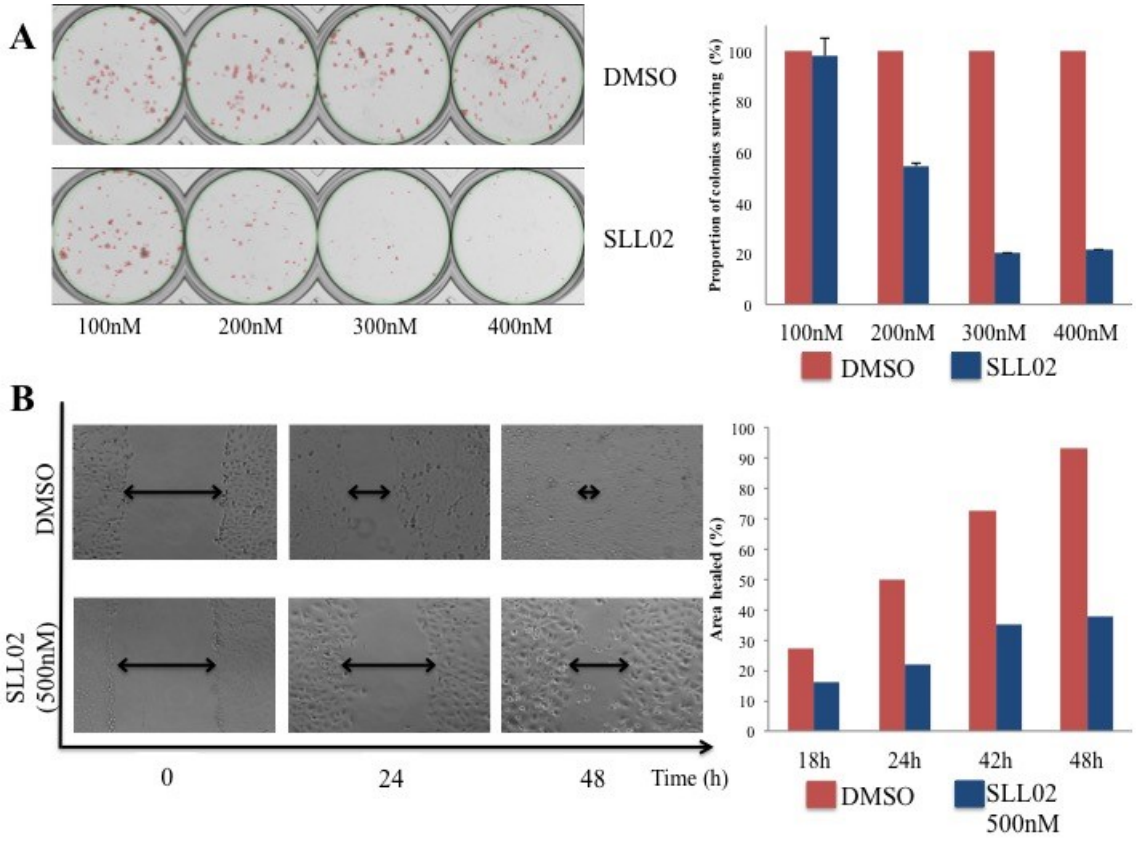
To investigate the ability of SLL02 to inhibit colony forming two hundred 8505c cells were seeded in a 12-well plate (Corning ©). The following day, treatment was started in triplicates at increasing concentration with a negative control for each concentration. Treatment with SLL02 resulted in a dose-response reduction of the number of colonies forming (Figure 8.2A, $P<0.001$). Cell motility was assessed using the wound-healing assay, where 8505c cells were treated with 500 nM SLL02 and compared with DMSO-treated cells. The cells' ability to migrate and heal the wound is shown in Figure 8.2B. After 48h, the control (DMSO) treated wounds completely closed, whereas the SLL02-treated wounds did not.

Figure 8.2: Effect of SLL02 on proliferation and motility

A: Clonogenic assay with on the left representative GelCount ® processed image of cells treated with DMSO (first row) and SLL02 (second row) with increasing concentration. On the right, graphical representation of SLL02 effect on colony formation. Each experiment, done in triplicate, was performed three times. Mean values and standard deviations were obtained from a total of nine values. The difference between DMSO and SLL02 is highly significant (dose-response analysis, $P<0.001$).

B: Wound healing assay. Left side: representative microscope pictures of plated 8505c cells at different time points of assay. The gap closes for untreated (DMSO) cells after 48 hours (1st row), while SLL02 inhibits “wound closure” (2nd row). On the right, graphical representation of wound

healing assay. Values on y-axis indicate percentage of gap remaining. .Each experiment was done in triplicate.



SLL02 inhibits* tumor growth *in vivo

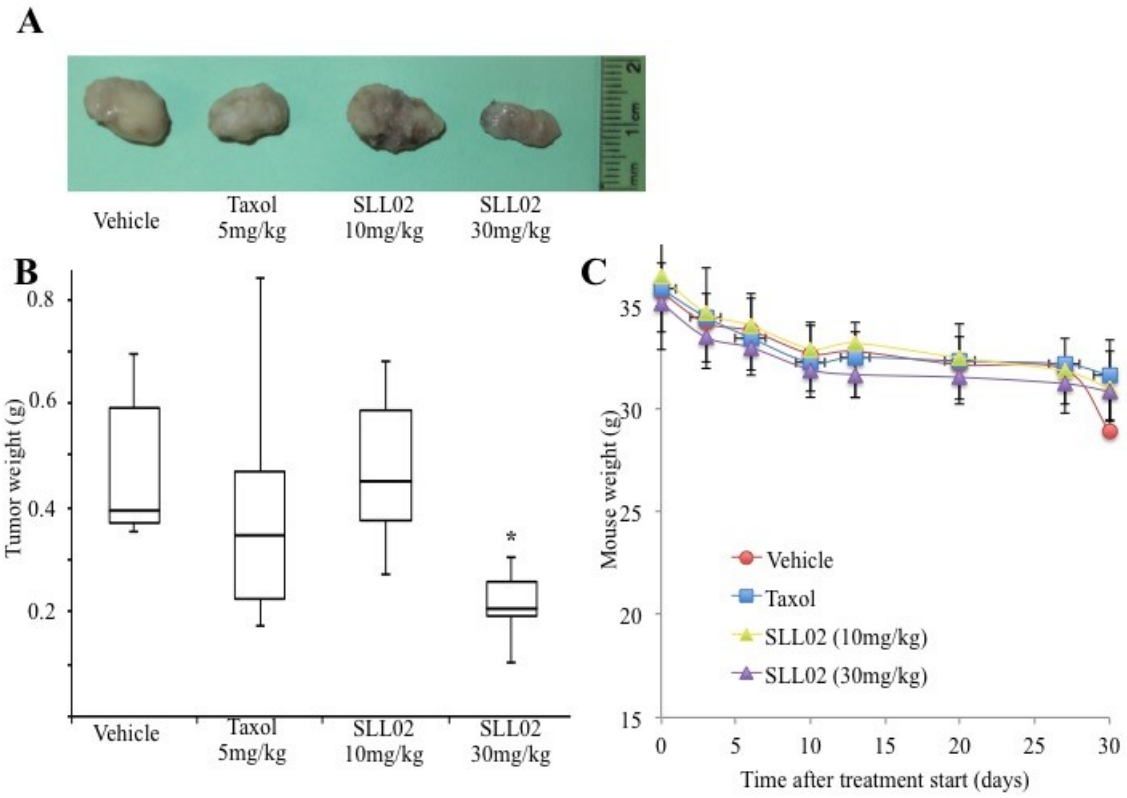
To further investigate the impact of SLL02 on cell proliferation, we tested the efficacy of SLL02 *in vivo*. 8505c cells were transplanted heterotopically into the subcutaneous tissue of RAG mice. Mice were given tri-weekly injections of either 10 or 30 mg kg^{-1} SLL02 or vehicle or taxol [218]. SLL02 at 30 mg kg^{-1} induced a significant inhibition of primary tumor growth as measured by tumor weight upon autopsy (P=0.023, Figure 8.3A and B). A dose of 10 mg kg^{-1} SLL02 did not impact on tumor growth in our model. As previously reported [234], we did not see any distant metastasis to the lungs and/or the liver upon necropsy in any mice in this heterotopic model. SLL02 was well tolerated, as no differences in body weight, animal behaviour or mortality were seen (Figure 8.3C).

Figure 8.3: In vivo experiment. 10'000'000 cells of 8505c cell line were injected s.c. in RAG mice. Mice were treated i.p. with either taxol or SLL02 for 30 days and sacrificed after this period.

A: Representative tumor for each study group after dissection.

B: Median (Interquartile range) shown for tumor weight upon dissection of mice. Tumors in the last group (DW 82 30mg) are significantly smaller (Kruskal-Wallis P=0.023).

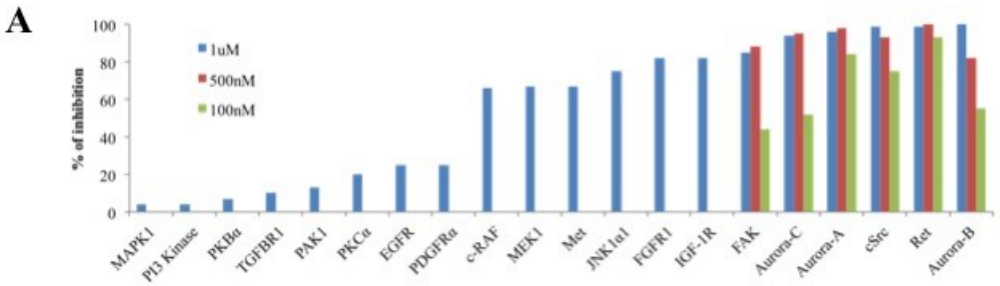
C: Mean (+SEM) mouse weight after start of treatment. No significant differences are seen (P>0.05).



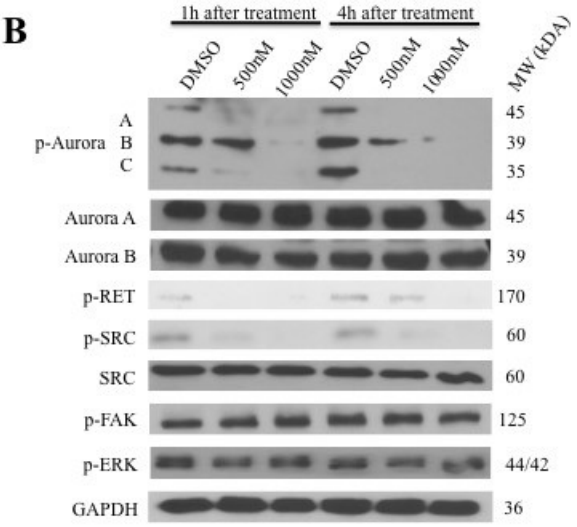
SLL02 inhibits phosphorylation of kinases in silico and in vitro. After proving in vitro and in vivo efficacy of our novel compound, we ought to elucidate the mechanism by which this occurred. SLL02 was first evaluated using an *in silico* kinase assay (Select Screen ®), showing inhibition Aurora A, B, and C kinases, as well as RET and SRC. For the ERK/MAPK and FAK kinase, no effect was seen (Figure 8.4A). These results were confirmed *in vitro* treating 8505c cells with SLL02 for 1h and 4h, respectively (Figure 8.4B). The quantity of p-Aurora A, B, and C diminished in a dose-response manner after 1h and 4h of treatment, with a stronger effect for the

latter. The levels of Aurora A and B, as well as GAPDH remained constant. SLL02 also induced inhibition of p-RET and p-SRC after 4 hours. For p-ERK/MAPK and p-FAK, no effect was seen. Together, these results suggest that SLL02 is capable of inhibiting multiple kinases.

Figure 8.4: (A) Kinase activity of SLL02 in SelectScreen ® assay. (B) Western blot shown relative p-Kinase levels after 1h and 4h of treatment with SLL02 in 8505c cells.



Kinase		100nM	500nM	1000nM
		% of inhibition		
Aurora	A	84	98	96
	B	55	82	100
	C	52	95	94
RET		93	100	99
SRC		76	93	99
FAK		44	88	83
ERK		*NA*	*NA*	4



Effect of SLL02 induces reduction of ALDH+ subpopulation. As increased phosphorylation of key kinases has been associated with epithelial-mesenchymal transition markers and increased cancer stem-cell like population in ATC [64, 66, 221], we further evaluated the effect of SLL02 on several cancer stem markers such as ALDH, CD144, CD24 and CD44 (Table 8.1) [220, 235]. Interestingly, treatment with SLL02 induced a dose-response shift of ALDH+ cell population, reducing the percentage of ALDH+ cells by roughly 90% at 200nM. At a concentration of 400nM, the percentage of ALDH+ positive cells was equivalent to those seen in the negative controls treated with diethylaminobenzaldehyde (DEAB) (Figure 8.5). DEAB treatment serves as a negative control as it annihilates ALDH activity. Importantly, the effect seen on ALDH+

activity is not biased by necrotic or apoptotic cells (without ALDH activity) as these cells were removed FACS before analysis of ALDH activity (see methods).

Table 8.1: SLL02's impact on cancer stem-like markers in thyroid carcinoma cell line 8505c

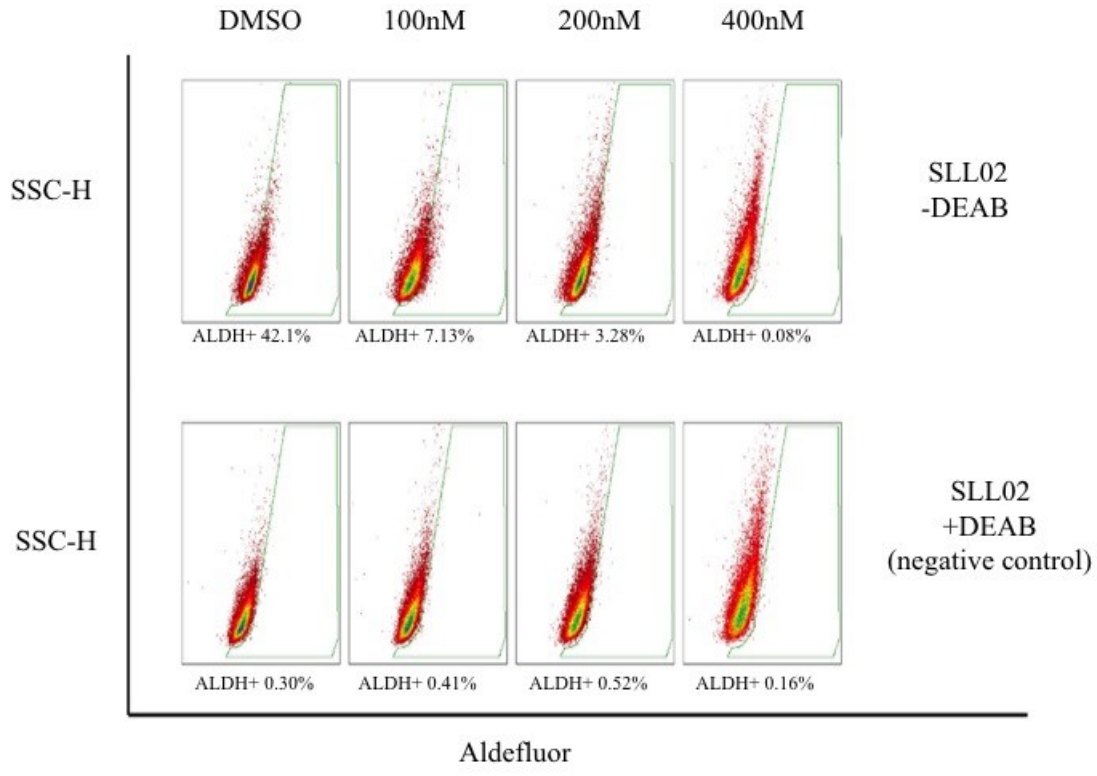
	DMSO	**SLL02**
CD44	99.87% ± 0.1%	99.87% ± 0.1%
CD24	99.87% ± 0.1%	99.87% ± 0.0%
CD133	0.2% ± 0.2%	0.7% ± 0.3%
ALDH	17.7% ± 2.4%	5.4% ±1.3%

Sub-confluent cells were exposed to 300nM SLL02 for 72h and then the percentage of total live cells expressing cancer stem cell-like markers were quantified using FACS analysis, as described in methods. Control cells were exposed to the vehicle alone. Shown are the average ± SD of at least 3 independent experiments each in duplicates.

Figure 8.5: FACS analysis of 8505c cells treated with SLL02. Aldehyde dehydrogenase (ALDH) activity – a surrogate marker for cancer stem cell population - is measured using the Aldefluor test.

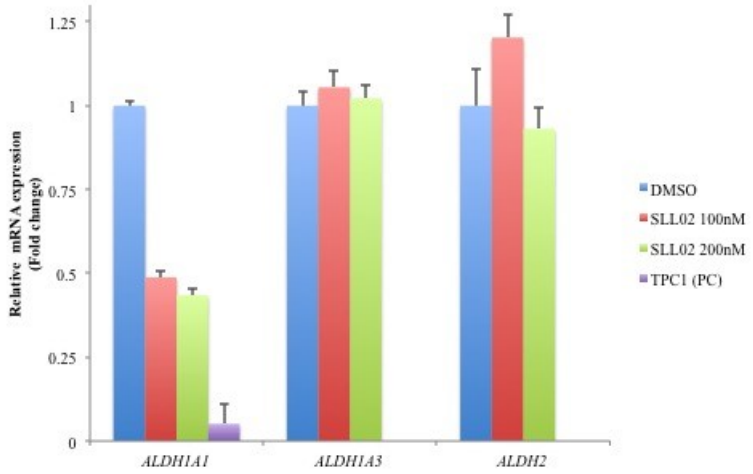
ALDH activity decreasing in a dose-response manner upon treatment with SLL02. DEAB treatment serves as a negative control as DEAB annihilates ALDH activity.

SSC-H: Side Scatter Pulse Height.



Previous studies have shown that the isoform *ALDH1A1* was responsible for most of ALDH activity in thyroid cancer and that its expression was higher in anaplastic thyroid cancer, compared to well-differentiated papillary thyroid cancer [221]. Treatment with SLL02 induced a dose-dependent decrease in mRNA levels of ALDH1A1 (Figure 8.6), while the positive control from a well-differentiated papillary thyroid cell line showed very low endogenous levels of *ALDH1A1*. For *ALDH1A3* and *ALDH2*, no significant change in mRNA expression was seen.

Figure 8.6: Fold change induced in mRNA ALDH isoforms expression upon treatment with SLL02. Negative control treated with DMSO. As positive control (PC), a papillary thyroid cell line was used (TPC1).



8.5. Discussion

Anaplastic thyroid carcinoma (ATC) is one of the most deadly human malignancies and is thought to result from progressive de-differentiation from follicular thyroid cells through accumulation of genetic and epigenetic events that drives carcinogenesis and/or proliferation of cancer stem cell-like subpopulations showing highly aggressive features [9, 219, 220]. In this study, we investigated the potential therapeutic benefit of SLL02, a novel purine-derived multi-kinase inhibitor. *In vitro*, we showed that SLL02 was inhibiting kinase activity of Aurora A, B and C, and to a lesser extent RET and SRC enzymes. This molecule was also found to induce cell differentiation reprogramming with reduction of ALDH+ cancer stem cell-like subpopulation.

Aurora kinases are members of a family of serine/threonine kinases. They are involved in regulation of multiple steps of mitosis, including centrosome duplication, chromosome alignment, and spindle checkpoint [236]. In particular, Aurora B is a chromosome passenger protein forming a complex with Survivin, an inner centromere protein (INCENP) and Borealin during anaphase. Aurora B is also required for histone phosphorylation during mitosis [236]. This kinase is extensively overexpressed both at the translational and transcriptional level in ATC tissues and cell lines, whereas it is always absent and rare in benign and papillary thyroid samples, respectively [223]. *In vivo*, Aurora B transfection resulted in polyploidy and aggressive tumor phenotype [237], while Aurora B knockdown by siRNA was shown to significantly

decrease tumor proliferation [223]. SLL02 treatment resulted in dramatic reduction of p-Aurora A, B, and C levels, thus preventing activation of downstream events.

To our knowledge, only a few other Aurora kinase inhibitors have been evaluated in thyroid cancer [223, 225]. A drawback of targeting one single pathway is the rapid emergence of drug resistance mechanisms, such as secondary mutation of target, enhanced drug inactivation by phase II enzymes, up-regulation of survival signals, and activation of compensatory pathways able to bypass signalling blockade [226]. The development of either multi-targeted protein kinase inhibitors or cocktails of selective antagonists can overcome this issue and provided us the rationale for the development of SLL02 [226]. SLL02 was shown not only to inhibit three members of the Aurora family, but also RET and SRC enzymes.

A further effect induced by SLL02 was a dramatic reduction in ALDH+ stem cell-like subpopulation. Previous studies have shown that ALDH activity, as measured by ALDEFLUOR test, was the most reliable stem cell marker in thyroid cancer [221, 238]. ALDH+ positive thyroid cells retained tumor initiating capacity and had unlimited replication potential [221]. For the particular cell line used in this study, similar results are reported for ALDEFLUOR baseline and negative controls, thus validating our results [235]. Although the exact mechanism by which SLL02 induced a dose-dependent ALDH+ decrease is not fully elucidated, activation of STAT3/RET has been associated with ALDH+ cancer stem cell-like population in colon cancer [239]. Treatment with SLL02 could hence achieve ALDH+ reduction possibly via transcriptional inhibition mediated by these kinases, including inhibition of p-RET. Indeed, we observed partial inhibition of *ALDH1A1* transcription upon treatment with SLL02.

The inhibition of the above described pathways and subpopulations resulted practically in decreased colony forming and cell locomotion *in vitro*. Our group has previously shown that focal adhesion, a critical event in cell migration and invasion, was regulated by the SRC-FAK pathway [62] and that purine derivatives were capable of inhibition of focal adhesion, explaining decreased locomotion [233]. The inhibition in colony forming seems more straight forward as SLL02 inhibited all three Aurora kinases, which are regulators of mitoses. These *in vitro* results were confirmed by significant tumor growth inhibition in a heterotopic mouse model. Importantly, ALDH inhibition was also shown to affect cancer cell proliferation and motility in lung cancer [227].

In conclusion, our preclinical data suggests SLL02 to be a promising new therapy in patients suffering from ATC. SLL02 showed inhibition of multiple kinases and ALDH activity *in vitro* that translated into significant tumor growth reduction *in vivo*. Clinical investigations using SLL02 alone and as an adjunct to standard of care may be a promising field to broaden the therapeutic possibilities against ATC.

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Authors' contributions:

Manuscript writing: GBM under KB, SDDS and MAAJ supervision. In vitro studies by GBM. FACS analysis by HY. In vivo studies by GBM and JS. Synthesis of compounds by DW under KB and MAAJ supervision. All authors substantially participated to the manuscript and approved the final version.

Disclosure statement

None of the authors reports a conflict of interest.

9. General Discussion and conclusion

Linking statements from manuscripts

The main goal of this thesis was to provide further understanding of signalling pathways affecting the behaviour and prognosis of follicular cell-derived thyroid cancer. In the first part, we compared tumors showing an aggressive metastatic phenotype to tumors without metastasis. We showed that the metastatic process, by which sessile cells become motile, become capable of invading local tissue, and then settle and proliferate, requires activation of many genes responsible for cell-cell interaction, cell survival, degradation of extracellular matrix, escape and modulation from physiological immune response. This is in accordance with now well-established concepts linked to the metastatic process [59, 240]. We also demonstrated a previously undescribed cooperation between two major cancer pathways, namely the BRAF/MAPK pathway and the WNT/b-catenin pathway, in the progression of thyroid cancer, thus supporting an intricate crosstalk that may contribute to a more aggressive phenotype seen in BRAF mutated tumors [134-136].

Therefore, in the first part of the thesis, we provided a comprehensive view and characterization of molecular mechanisms implicated in the metastatic process of thyroid cancer. In the second part, we documented how genetic and epigenetic events can actually out-weight previous case control studies investigating chemopreventive properties of diet supplements. Using the example of vitamin D, we showed that surrogate markers or measures of vitamin D status, such as sun exposure, dietary intake, and/or blood measurement, hardly reflect the availability and effect of vitamin D at the local level, that is, its direct effect on thyroid cells. Vitamin D acts a nuclear transcription factor with complex metabolism and regulation processes, thus rendering the estimates of vitamin D status very unlikely to be accurately linked – more than by chance alone – to biological processes.

In the final part of the thesis, we presented a novel small molecule that targets multiple kinases in a model of anaplastic thyroid cancer. As described above, the complexity and multitude of biological regulations and compensation mechanisms provide the rationale for novel future drugs targeting multiple key processes in malignant cells, thus rendering the development of acquired resistance less likely. Anaplastic thyroid cancer is characterized by ultimate de-differentiation of follicular cell derived thyroid neoplasm. Those tumors are more likely to present an accumulation of multiple mutations and drivers oncogenic events rendering the success of a "single pathway"

inhibitor less likely. Furthermore, in addition to the stochastic model of multi-hit carcinogenesis and tumor progression, recent evidence support the hierarchical model of cancer stem cell-like subpopulations [241].

Conclusions

The conclusions of this thesis work are:

1. Metastatic process involves differential expression of a plethora of genes, allowing cell motility, survival in new environments, and proliferation.
2. Due to the complexity of cancer initiation and progression, comprehensive and in-depth studies of molecular mechanism related to the host and the tumor are necessary
3. Novel therapeutics should be designed to tackle the complexity and multitude of molecular changes seen in cancer.

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11. Annexes
11.1. Informed consent forms

Sir Mortimer B. Davis Jewish General Hospital
Department of Oncology and Head and Neck Surgery
Montreal, QC, Canada

CONSENT FORM

Molecular profiling of metastatic non-medullary thyroid cancer for discovery of innovative prognostic and therapeutic biomarkers

1. OVERVIEW

For research purposes, we would like to use a small sample of your thyroid gland, which was removed many years ago. This small sample is actually in storage facilities at our hospital and not being used. By signing this consent form, you allow us to use a small amount of your thyroid gland for research. Further details about the research purposes and goals are provided below. You do not have to take part in this research if you do not want to.

2. DEFINITIONS

Genes are the basis for hereditary traits, such as the color of our eyes or our blood group type. The information of the genes is coded into DNA. Based on this genetic information, cells build different sorts of proteins, which are the basis of human tissues. An abnormality in the genetic code, even in a single letter of code is incorrect, can favor the development cancer.

In biomedical research, a biological sample includes all organs, tissues or other biological substances (for example blood, urine, etc) that can be sampled from a person. In order for research to advance, scientists need biological samples coming from both normal and diseased organs and tissues.

In this study, the biological pathways and process, critical for tumor development, progression and spread will be investigated in thyroid cancer in order to analyze the genes and proteins expressed in thyroid cancer.

The study will be using a technology called Tissue microarray (TMA). TMAs are very good for the investigation of protein expression in different kinds of cancers.

3. OBJECTIVES

The aim of this study is to understand the causes of thyroid cancer, or the underlying mechanisms that characterize those cancers and their metastatic potentials, in order to eventually develop new treatments for their cure. In the future, it may be possible that this research will result in the development of new clinical tools, such as early detection tests, new treatments, etc. All biological samples and clinical information collected from you will be solely used for this research project.

You have been asked to provide us with your tissue biological sample because you are a patient who underwent thyroid surgery during the last 10 years at Jewish General Hospital by Dr. Black, Dr. Hier, and Dr. Payne, and we would like to obtain a very small tissue specimen of your already resected tumor tissue. The tissue specimen will be studied through Tissue Microarray (TMA) of formalin-fixed and paraffin-embedded tissue blocks at Jewish General Hospital pathology laboratory.

In order to conduct the research, we may need information from your chart. Only the primary investigators (Dr. Gregoire Morand and Sabrina Daniela da Silva) who are the authorized personnel of Dr. Black, Dr. Hier, Dr. Mlynarek, and Dr. Payne would consult your medical files to obtain vital information in order to properly analyze research results. These might include information such as your age, your gender, your diagnosis, your actual state of health, your previous health history, as well as treatments you have received and how you responded to these treatments. This information is entered in a database that respects all confidentiality and privacy laws as determined by provincial and federal legislation.

4. BIOLOGICAL SAMPLES

4.1 Identification of biological samples

Your pathological preserved tissue is assigned with a unique pathological number, and this number will allow us to ensure confidentiality when linking clinical and research information.

4.2 Specimen conservation

Your pathological specimen is already stored at the Jewish General Hospital pathology laboratory since the day of your surgery. We would like to obtain very small sample from for this research (1mm). Once the research is completed, your tissue will undergo the usual hospital procedure for disposing biological material.

5. BENEFITS

There is no direct benefit to you by participating in this study. The research done with your sample will be used to further contribute to our knowledge in thyroid cancer, with the goal of developing strategies primarily for diagnosis, and secondarily for prevention, treatment and/or cure of this disease.

6. RISKS

There is no risk to your health by participating in this study because we are using a sample of your already preserved tissue.

7. SOCIAL AND ECONOMIC RISKS

The authorities of the repository have taken strict measures related to confidentiality, privacy and security of the samples and data within the laboratory. No information will be released to your family, insurers or employers without your written consent, unless required by law.

8. CONFIDENTIALITY OF TISSUE AND DATA

While you take part in this research study, the researcher in charge and study staff will collect and store personal identifiable information about you in a file for the purpose of the research study. Only information necessary for the research study will be collected.

All the information collected about you during the study will remain confidential within the limits of the Law. To protect your identity, your name and identifying information will be replaced with a code (numbers and or letters), the link between the code and your identity will be held by the researcher in charge of the study. No information that discloses your identity will be allowed to leave the institution. Your study information will be kept for 10 years by the researcher in charge of the study. Your sample will be kept for 2 months and any remaining sample will be sent back to Pathology.

The study information may also be used for other reasons related to the study or to help in the development of future studies. There are no plans to compensate you for any products developed from this research.

The study information could be printed/published in medical journals or shared with other people at scientific meetings, but your identity will not be revealed.

For the purpose of monitoring this research, your research study file as well as your medical records identifying you could be checked by a person authorized by the Research Ethics Committee of the Jewish General Hospital.

9. COST AND COMPENSATION:
You will not have to pay nor will you be paid for your participation in this study.

10. COMMERCIALIZATION
Your biological sample and the associated data will not be sold and will only be used for research this purpose only. The research may result in the creation of new commercial products but you will not be entitled to any financial benefits from these products.

11. FREEDOM OF PARTICIPATION
Your contribution to this research is done on a completely voluntary basis and your choice will not in any way change the way you are treated at the JGH. You can change your mind about participating at any time.

Should you withdraw your consent, all biological samples and information collected for the purpose of the research up to the point of your withdrawal may be used in order to preserve the scientific integrity of the study.

12. PROBLEMS OR QUESTIONS

If you have any questions concerning the tissue repository, please contact the primary investigator: Dr. Sabrina Daniela da Silva at 514-299-2188 or Dr Gregoire Morand at 514 746 1757 or the clinical coordinators: Dr.Hier, Dr. Black or Dr. Mlynarek or Dr. Payne at 514-340-8222 ext 5387

For additional information or if you have questions about your rights as a research participant, you may call the Jewish General Hospital Local Commissioner of Complaints & Quality of Services, Rosemary Steinberg, at (514) 340-8222 ext. 5833.

Molecular profiling of metastatic non-medullary thyroid cancer for discovery of innovative prognostic and therapeutic biomarkers

Statement of Consent

I have read the consent form entitled 'Molecular profiling of metastatic non-medullary thyroid cancer for discovery of innovative prognostic and therapeutic biomarkers'

I had the opportunity to ask questions and the answers provided are satisfactory.

I understand that my participation in this project is entirely voluntary, and that I remain free to withdraw my participation at any time. It is understood that my decisions will not in any way affect the quality of care I will receive. By signing this consent form I do not give up my legal rights, nor do I relieve the researchers, the hospital, or funding organizations from their professional and legal obligations.

Therefore, I consent to the following points:

I accept that part of the tissue material obtained from me will be used for research purposes, and I authorize access to my medical record in order to obtain information necessary for the appropriate use of this sample.

❑Yes ❑ No

Signature: _____ Date: _____

Name of Participant: _____

Consent form administered and explained over the phone by:

Signature: _____ Date: _____

Name of Investigator: _____

French consent forms were also available so that French-speaking patients could provide their consent

11.2. Supplemental Tables for Chapter 6

Suppl. Table 1: Trimming and alignment statistics for each sample

Sample	9-N0	23-N1	4-N0	2-N0	27-N0	15-N0	1-N1	35-N0	14-N1
Raw reads	9101590	7918015	9700512	8257587	8541120	9916565	8471210	7453352	1010704
Surviving reads	8776943	7689403	9383881	7984433	8286689	9607862	8197661	7177150	9777851
%	96.43308	97.11275	96.73593	96.69209	97.02110	96.88699	96.77082	96.29426	96.74293
Aligned read	8426183	7453008	9079966	7746896	7987701	9291993	7904829	6938322	9475945
%	96.00361	96.92570	96.76130	97.02499	96.39195	96.71239	96.42785	96.67238	96.91234
Alternative	6131102	5524700	6585399	4199608	4346067	6869937	7793467	7923205	6233274
%	7.276250	7.412710	7.252669	5.421019	5.440948	7.393394	9.859121	11.41948	6.577997
rRNA reads	0	0	0	0	0	0	0	0	0
%	0	0	0	0	0	0	0	0	0
Coverage	61	53	70	58	49	73	49	52	75
Exonic Rate	0.821054	0.882224	0.887841	0.866852	0.765138	0.863831	0.849547	0.867161	0.895813
Genes	22203	22139	22577	22170	21681	23267	22123	21873	23326

34-N0	20-N0	5-N1	10-N1	38-N1	41-C	7-N0	39-N1	24-N1	31-C	29-C
9440928	8341892	7983828	8366805	7884393	9183405	7053461	9187626	1051065	7437070	8545544
9089437	8062949	7751435	8100040	7551419	8873177	6845131	8903042	1016818	7210735	8285308
96.27694	96.65612	97.08920	96.81162	95.77679	96.62186	97.04641	96.90252	96.74171	96.95667	96.95471
8776197	7774230	7504139	7844396	7248171	8560478	6547023	8584470	9821755	6976355	7995090
96.55380	96.41918	96.80967	96.84392	95.98423	96.47590	95.64496	96.42175	96.59295	96.74956	96.49720
5975323	7820867	4903715	8211978	5820364	5919001	6030139	6226722	6768976	3328871	3459381
6.808555	10.05998	6.534680	10.46859	8.030113	6.914334	9.210505	7.253472	6.891819	4.771647	4.326881
0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0
59	46	54	59	54	63	48	55	73	54	56
0.862194	0.815857	0.868453	0.866845	0.894643	0.8846	0.872904	0.849827	0.869688	0.886206	0.870174
22645	21789	23221	22672	21622	23099	23231	23154	22583	22274	23294

22-N1	25-N0	21-N1	17-C
8532170	6234499	8844986	8446676
8266605	6008376	8516067	8216844
96.88748	96.37304	96.28129	97.27902
8012824	5740604	8150031	7927594
96.93005	95.54334	95.70181	96.47980
6932986	1126505	8508197	5521199
8.652361	19.62346	10.43946	6.964532
0	0	0	0
0	0	0	0
53	22	37	52
0.874645	0.619527	0.750814	0.849896
23139	21211	21750	23898

Suppl. Table 2: Details about analysis parameters and software versions

	"type"	"name"	"value"
1	"default"	"projectName"	"RNAseq"
2	"default"	"rawReadDir"	"/mnt/parallel_scratch_mp2_wipe_on_august_2014/bourque/bourque_group/projects/malaoui_RNAseq_metastatic_thyroid_cancer_PRJBFX-692/raw_reads/"
3	"default"	"libraryType"	"paired"
4	"default"	"report.title"	"malaoui_RNAseq_metastatic_thyroid_cancer"
5	"default"	"cycleNumber"	"100"
6	"default"	"clusterSubmitCmd"	"qsub"
7	"default"	"clusterSubmitCmdSuffix"	" | grep \""[0-9]\"""
8	"default"	"clusterWalltime"	"-l walltime=120:00:0"
9	"default"	"clusterCPU"	"-l nodes=1:ppn=1"
10	"default"	"clusterOtherArg"	"-V -m ae -M $JOB_MAIL -W umask=0002"
11	"default"	"clusterQueue"	"-q qwork"
12	"default"	"clusterWorkDirArg"	"-d"
13	"default"	"clusterOutputDirArg"	"-j oe -o"
14	"default"	"clusterJobNameArg"	"-N"
15	"default"	"clusterCmdProducesJobId"	"true"
16	"default"	"clusterDependencyArg"	"-W depend=afterok:"
17	"default"	"clusterDependencySep"	":"
18	"default"	"extraJavaFlags"	"-XX:ParallelGCThreads=4"
19	"default"	"tmpDir"	"\\\$LSCRATCH"
20	"default"	"moduleVersion.bed2wig"	"mugqic/ucsc/20130924"
21	"default"	"moduleVersion.bedtools"	"mugqic/bedtools/2.17.0"
22	"default"	"moduleVersion.bowtie"	"mugqic/bowtie2/2.2.2"
23	"default"	"moduleVersion.bwa"	"mugqic/bwa/0.7.9a"
24	"default"	"moduleVersion.cufflinks"	"mugqic/cufflinks/2.1.1"
25	"default"	"moduleVersion.cranR"	"mugqic/R/3.1.0"
26	"default"	"moduleVersion.java"	"mugqic/java/openjdk-jdk1.7.0_60"
27	"default"	"moduleVersion.picard"	"mugqic/picard/1.108"
28	"default"	"moduleVersion.rnaseqc"	"mugqic/rnaseqc/1.1.7"
29	"default"	"moduleVersion.samtools"	"mugqic/samtools/0.1.19"
30	"default"	"moduleVersion.tools"	"mugqic/tools/1.10"

31	"default"	"moduleVersion.tophat"	"mugqic/tophat/2.0.11"
32	"default"	"moduleVersion.trimmomatic"	"mugqic/trimmomatic/0.32"
33	"default"	"moduleVersion.python"	"mugqic/python/2.7.6"
34	"default"	"referenceFasta"	"/mnt/parallel_scratch_mp2_wipe_on_august_2014/bourque/bourque_group/analyste/genomes/Homo_sapiens/hg19/fasta/hg19.fasta"
35	"default"	"referenceSequenceDictionary"	"/mnt/parallel_scratch_mp2_wipe_on_august_2014/bourque/bourque_group/analyste/genomes/Homo_sapiens/hg19/fasta/hg19.dict"
36	"default"	"chromosomeSizeFile"	"/mnt/parallel_scratch_mp2_wipe_on_august_2014/bourque/bourque_group/analyste/genomes/Homo_sapiens/hg19/hg19.chromsize.txt"
37	"default"	"referenceGtf"	"/mnt/parallel_scratch_mp2_wipe_on_august_2014/bourque/bourque_group/analyste/genomes/Homo_sapiens/hg19/annotations/transcripts_ensembl.gtf"
38	"default"	"referenceEnsemble2symbol"	"/mnt/parallel_scratch_mp2_wipe_on_august_2014/bourque/bourque_group/analyste/genomes/Homo_sapiens/hg19/annotations/gene2geneName.tsv"
39	"default"	"geneSizeFile"	"/mnt/parallel_scratch_mp2_wipe_on_august_2014/bourque/bourque_group/analyste/genomes/Homo_sapiens/hg19/annotations/gene_lengths.tsv"
40	"default"	"ribosomalFasta"	"/mnt/parallel_scratch_mp2_wipe_on_august_2014/bourque/bourque_group/analyste/genomes/Homo_sapiens/hg19/rnaseqc/human_all_rRNA.fasta"
41	"default"	"goAnnotation"	"org.Hs.eg.db"
42	"default"	"igvGenome"	"hg19"
43	"samToFastq"	"extraJavaFlags"	"-XX:ParallelGCThreads=1 -Dsamjdk.use_async_io=true -Dsamjdk.buffer_size=4194304"
44	"samToFastq"	"samToFastqRam"	"30G"
45	"trim"	"nbThreads"	"24"
46	"trim"	"minQuality"	"30"
47	"trim"	"minLength"	"32"
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49	"trim"	"clipSettings"	":2:30:15"
50	"trim"	"clusterWalltime"	"-l walltime=24:00:0"

51	"align"	"referenceFasta"	"/mnt/parallel_scratch_mp2_wipe_on_august_2014/bourque/bourque_group/analyste/genomes/Homo_sapiens/hg19/fasta/bowtie2/alt/hg19.fa.fa"
52	"align"	"strandInfo"	"fr-firststrand"
53	"align"	"platform"	"ILLUMINA"
54	"align"	"TBInstitution"	"McGill University and Genome Quebec Innovation Centre"
55	"align"	"TBAlnThreads"	"24"
56	"align"	"clusterWalltime"	"-l walltime=120:00:0"
57	"align"	"transcriptomeIndex"	"/mnt/parallel_scratch_mp2_wipe_on_august_2014/bourque/bourque_group/analyste/genomes/Homo_sapiens/hg19/annotations/transcripts_ensembl_index/transcripts_ensembl"
58	"mergeFiles"	"mergeRam"	"30G"
59	"mergeFiles"	"mergeRecInRam"	"6750000"
60	"mergeFiles"	"extraJavaFlags"	"-XX:ParallelGCThreads=24 -Dsamjdk.use_async_io=true"
61	"mergeFiles"	"clusterWalltime"	"-l walltime=24:00:0"
62	"sortSam"	"sortRam"	"30G"
63	"sortSam"	"sortRecInRam"	"6750000"
64	"sortSam"	"extraJavaFlags"	"-XX:ParallelGCThreads=24 -Dsamjdk.use_async_io=true"
65	"sortSam"	"clusterWalltime"	"-l walltime=24:00:0"
66	"reorderSam"	"reorderRam"	"30G"
67	"reorderSam"	"reorderRecInRam"	"6750000"
68	"reorderSam"	"extraJavaFlags"	"-XX:ParallelGCThreads=24 -Dsamjdk.use_async_io=true"
69	"reorderSam"	"clusterWalltime"	"-l walltime=24:00:0"
70	"markDup"	"markDupRam"	"30G"
71	"markDup"	"markDupRecInRam"	"3500000"
72	"markDup"	"extraJavaFlags"	"-XX:ParallelGCThreads=24 -Dsamjdk.use_async_io=true"
73	"markDup"	"clusterWalltime"	"-l walltime=48:00:0"
74	"wiggle"	"clusterWalltime"	"-l walltime=12:00:0"
75	"htseq"	"clusterWalltime"	"-l walltime=24:00:0"
76	"htseq"	"options"	"-m intersection-nonempty"
77	"fpkm"	"cufflinksThreads"	"24"
78	"fpkm"	"cufflinksMaxFargs"	"1000000"
79	"fpkm"	"clusterWalltime"	"-l walltime=48:00:0"
80	"cuffdiff"	"numThreads"	"24"
81	"cuffdiff"	"options"	"-u --mask-file /mnt/parallel_scratch_mp2_wipe_on_august_2014/bourque/bourque_group/analyste/genomes/Homo_sapiens/hg19/annotations/hg19_rRNA_tRNA_chrM.gtf "

82	"cuffdiff"	"clusterWalltime"	"-l walltime=48:00:0"	
83	"cuffcompare"	"clusterWalltime"	"-l walltime=2:00:0"	
84	"rnaQc"	"clusterWalltime"	"-l walltime=120:00:0"	
85	"rnaQc"	"clusterQueue"	"-q qfat256"	
86	"rnaQc"	"extraJavaFlags"	"-XX:ParallelGCThreads=4 -Dsamjdk.use_async_io=true"	
87	"rnaQc"	"metricsRam"	"240G"	
88	"rnaQc"	"topTranscript"	"1000"	
89	"metrics"	"clusterWalltime"	"-l walltime=5:00:0"	
90	"saturation"	"optionR"	"1"	
91	"saturation"	"threadNum"	"19"	
92	"saturation"	"clusterWalltime"	"-l walltime=24:00:0"	
93	"saturation"	"clusterQueue"	"-q qfat256"	
94	"diffExpress"	"clusterWalltime"	"-l walltime=10:00:0"	
95	"diffExpress"	"cuffRescolumns"	"""3 13"" "	
96	"diffExpress"	"dgeRescolumns"	"""2 6"""	
97	"goseq"	"clusterWalltime"	"-l walltime=10:00:0"	
98	"goseq"	"referenceUCSCname"	"hg19"	
99	"goseq"	"geneIdType"	"geneSymbol"	
100	"downstreamAnalyses"	"clusterWalltime"	"-l walltime=00:30:0"	
101	"report"	"designFile"	"design.tsv"	
102	"report"	"clusterWalltime"	"-l walltime=00:30:0"	

Suppl. Table 3: Primers sequences used for qPCR confirmation

	Primer sequence	
Gene	F 5' to 3'	R 5' to 3'
HRPT1	GAA CGT CTT GCT CGA GAT GTG A	TCC AGC AGG TCA GCA AAG AAT
FN1	ACC TCG GTG TTG TAA GGT GG	CCA TAA AGG GCA ACC AAG AG
SERPINA1	ACG AGA CAG AAG ACG GCA TT	CCA CTG CTT AAA TAC GGA CGA
CLDN10	GCA TGT AGA GGA CTT ATG ATC GC	TCC GAC TTT GGT ACA CTT CAT TC
CD55	GGT GCA ACC ATC TCC TTC TC	TGG TGG TGC TGG ACA ATA AA
CD36	ACA ATT TGC AAA ACG GCT GC	GGT CCC AGT CTC ATT AAG CCA
RSPO4	AAG GAA GAA GCA AGT GGG CA	GGA ACA GCC GTT CTC CTC TG

Suppl. Table 4: Pairwise sample correlation analysis

29-C	9-N0	23-N1	4-N0	2-N0	27-N0	15-N0	1-N1	35-N0	14-N1	Vs.
0.9448431	0.9323788	0.9699372	0.9452421	0.9383054	0.9312586	0.9438818	0.9544531	0.9530788	1	14-N1
0.9721874	0.9605845	0.9556246	0.9597354	0.9638022	0.9567485	0.9772741	0.9478442	1	0.9530788	35-N0
0.9493635	0.9390478	0.9709263	0.9554381	0.9380548	0.9447096	0.9416341	1	0.9478442	0.9544531	1-N1
0.9712372	0.9551835	0.9471149	0.9578659	0.9604017	0.9543217	1	0.9416341	0.9772741	0.9438818	15-N0
0.9578643	0.9644130	0.9406660	0.9529540	0.9651969	1	0.9543217	0.9447096	0.9567485	0.9312586	27-N0
0.9621686	0.9578212	0.9426022	0.9552188	1	0.9651969	0.9604017	0.9380548	0.9638022	0.9383054	2-N0
0.9589580	0.9519526	0.9547956	1	0.9552188	0.9529540	0.9578659	0.9554381	0.9597354	0.9452421	4-N0
0.9475985	0.9427588	1	0.9547956	0.9426022	0.9406660	0.9471149	0.9709263	0.9556246	0.9699372	23-N1
0.9582649	1	0.9427588	0.9519526	0.9578212	0.9644130	0.9551835	0.9390478	0.9605845	0.9323788	9-N0
1	0.9582649	0.9475985	0.9589580	0.9621686	0.9578643	0.9712372	0.9493635	0.9721874	0.9448431	29-C
0.9805451	0.9647383	0.9483704	0.9610956	0.9709729	0.9633518	0.9757087	0.9428867	0.9767106	0.9435690	31-C
0.9469023	0.9431183	0.9784962	0.9510831	0.9409056	0.9405638	0.9434339	0.9669372	0.9554875	0.9785676	24-N1
0.9531658	0.9396529	0.9647329	0.9481961	0.9353231	0.9374270	0.9373412	0.9626561	0.9488152	0.9719189	39-N1
0.9547970	0.9429961	0.9396449	0.9404425	0.9397085	0.9415102	0.9372519	0.9396014	0.9433518	0.9360093	7-N0
0.9839646	0.9627587	0.9470307	0.9597214	0.9654793	0.9607992	0.9667800	0.9481029	0.9708540	0.9412518	41-C
0.9252919	0.9268739	0.9461382	0.9393095	0.9204801	0.9316174	0.9251881	0.9462103	0.9305770	0.9398816	38-N1
0.9515221	0.9403132	0.9769698	0.9604753	0.9419443	0.9396263	0.9442085	0.9706387	0.9544875	0.9729734	10-N1
0.9533939	0.9442023	0.9434941	0.9366703	0.9416644	0.9430742	0.9333370	0.9433318	0.9469142	0.9534854	5-N1
0.9574170	0.9584500	0.9504099	0.9705174	0.9525257	0.9596496	0.9546773	0.9601213	0.9588576	0.9382128	20-N0
0.9663058	0.9575411	0.9459983	0.9637476	0.9615578	0.9568601	0.9651842	0.9515750	0.9642833	0.9444950	34-N0
0.9770498	0.9538428	0.9512395	0.9520307	0.9543164	0.9545034	0.9527662	0.9546022	0.9592376	0.9466108	17-C
0.9359446	0.9215791	0.9473768	0.9374594	0.9168775	0.9286940	0.9252384	0.9715844	0.9286579	0.9308275	21-N1
0.9061934	0.8884393	0.9006110	0.9209003	0.8951586	0.9044584	0.9078876	0.9293288	0.8988907	0.8873918	25-N0
0.9568660	0.9374151	0.9619718	0.9516327	0.9370069	0.9406832	0.9410889	0.9713612	0.9502876	0.9662489	22-N1

17-C	34-N0	20-N0	5-N1	10-N1	38-N1	41-C	7-N0	39-N1	24-N1	31-C
0.9466108	0.9444950	0.9382128	0.9534854	0.9729734	0.9398816	0.9412518	0.9360093	0.9719189	0.9785676	0.9435690
0.9592376	0.9642833	0.9588576	0.9469142	0.9544875	0.9305770	0.9708540	0.9433518	0.9488152	0.9554875	0.9767106
0.9546022	0.9515750	0.9601213	0.9433318	0.9706387	0.9462103	0.9481029	0.9396014	0.9626561	0.9669372	0.9428867
0.9527662	0.9651842	0.9546773	0.9333370	0.9442085	0.9251881	0.9667800	0.9372519	0.9373412	0.9434339	0.9757087
0.9545034	0.9568601	0.9596496	0.9430742	0.9396263	0.9316174	0.9607992	0.9415102	0.9374270	0.9405638	0.9633518
0.9543164	0.9615578	0.9525257	0.9416644	0.9419443	0.9204801	0.9654793	0.9397085	0.9353231	0.9409056	0.9709729
0.9520307	0.9637476	0.9705174	0.9366703	0.9604753	0.9393095	0.9597214	0.9404425	0.9481961	0.9510831	0.9610956
0.9512395	0.9459983	0.9504099	0.9434941	0.9769698	0.9461382	0.9470307	0.9396449	0.9647329	0.9784962	0.9483704
0.9538428	0.9575411	0.9584500	0.9442023	0.9403132	0.9268739	0.9627587	0.9429961	0.9396529	0.9431183	0.9647383
0.9770498	0.9663058	0.9574170	0.9533939	0.9515221	0.9252919	0.9839646	0.9547970	0.9531658	0.9469023	0.9805451
0.9666926	0.9692104	0.9612971	0.9462613	0.9475643	0.9250963	0.9816496	0.9503900	0.9438439	0.9456973	1
0.9520475	0.9468258	0.9456220	0.9509894	0.9754568	0.9506666	0.9464709	0.9428284	0.9759137	1	0.9456973
0.9647703	0.9470646	0.9461648	0.9641084	0.9711572	0.9484751	0.9508299	0.9526443	1	0.9759137	0.9438439
0.9712365	0.9486946	0.9386294	0.9568802	0.9427113	0.9176777	0.9604402	1	0.9526443	0.9428284	0.9503900
0.9779571	0.9691661	0.9602269	0.9542671	0.9494688	0.9257275	1	0.9604402	0.9508299	0.9464709	0.9816496
0.9312708	0.9281662	0.938782	0.9232485	0.9487847	1	0.9257275	0.9176777	0.9484751	0.9506666	0.9250963
0.9551207	0.9521781	0.9536859	0.9527182	1	0.9487847	0.9494688	0.9427113	0.9711572	0.9754568	0.9475643
0.9686461	0.9476819	0.9409491	1	0.9527182	0.9232485	0.9542671	0.9568802	0.9641084	0.9509894	0.9462613
0.9542843	0.9653477	1	0.9409491	0.9536859	0.938782	0.9602269	0.9386294	0.9461648	0.9456220	0.9612971
0.9623909	1	0.9653477	0.9476819	0.9521781	0.9281662	0.9691661	0.9486946	0.9470646	0.9468258	0.9692104
1	0.9623909	0.9542843	0.9686461	0.9551207	0.9312708	0.9779571	0.9712365	0.9647703	0.9520475	0.9666926
0.9447643	0.9387688	0.9496213	0.9313786	0.9496071	0.9293525	0.9360357	0.9292679	0.9490921	0.9453833	0.9241456
0.9057011	0.9203733	0.9298440	0.8869819	0.9056819	0.8882811	0.9042563	0.8917910	0.8969522	0.8920116	0.9016185
0.9642995	0.9561272	0.9544359	0.9599453	0.9736788	0.9442970	0.9544017	0.9468218	0.9745184	0.9686961	0.9452648

22-N1	25-N0	21-N1
0.9662489	0.8873918	0.9308275
0.9502876	0.8988907	0.9286579
0.9713612	0.9293288	0.9715844
0.9410889	0.9078876	0.9252384
0.9406832	0.9044584	0.9286940
0.9370069	0.8951586	0.9168775
0.9516327	0.9209003	0.9374594
0.9619718	0.9006110	0.9473768
0.9374151	0.8884393	0.9215791
0.9568660	0.9061934	0.9359446
0.9452648	0.9016185	0.9241456
0.9686961	0.8920116	0.9453833
0.9745184	0.8969522	0.9490921
0.9468218	0.8917910	0.9292679
0.9544017	0.9042563	0.9360357
0.9442970	0.8882811	0.9293525
0.9736788	0.9056819	0.9496071
0.9599453	0.8869819	0.9313786
0.9544359	0.9298440	0.9496213
0.9561272	0.9203733	0.9387688
0.9642995	0.9057011	0.9447643
0.9608879	0.9432511	1
0.9197312	1	0.9432511
1	0.9197312	0.9608879

Suppl. Table 5: *available on request: gregoire.morand@mail.mcgill.ca (file too voluminous)*