S446 MOLECULAR-GENETIC CHARACTERISTICS OF THYROID TUMORS
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Differential diagnostics of nodular thyroid neoplasms is one of the most acute problems in thyroidology. Currently new molecular markers to distinguish between benign and malignant thyroid tumors are being searched. One way is to investigate intra- and extracellular signaling pathways, the deregulation of which leads to thyroid cell transformation. The aim of this study was to determine somatic mutations in BRAF gene, expression and activity of integrins, angiogenic factors, GSTP and microRNA in benign and malignant tumors for their differential diagnostics. In this study 120 samples of benign nodular neoplasms and 112 samples of papillary thyroid cancer (PTC) were analyzed. Somatic mutation V600E in BRAF oncogene was detected in 70% of all PTC cases. However, the mutation was not found in the benign samples. The identification of this mutation enables us not only to diagnose papillary thyroid cancer but also to prescribe adequate treatment including target therapy at the post-surgical stage. The gene expression and activity of the GSTP enzyme involved in hormone metabolism and c-Jun signaling pathways were different in PTC and benign nodular neoplasms. The results showed that GSTP can be used as cancer-specific marker for thyroid neoplasms with 83-88% sensitivity, 70-80% specificity and 76-84% diagnostic accuracy. Expression analysis for integrins α2, α5, αv, α9, β1, β3 and angiogenic factors IL-8, angiogenin and VEGF in normal and tumor thyroid tissues from patients with cancer and benign nodular neoplasms revealed heterogeneity in the expression of these genes, with higher expression being observed in malignant tumors. These indicators are important for the prognosis for the disease. We also examined the expression of miR 21, 221, 222, 155 in different types of thyroid neoplasms and found that it was increased in the cancer samples, which could be used for diagnostic purposes. Thus, the studied molecular markers could be used for differential diagnostics of benign and malignant thyroid neoplasms and the treatment approach.
EXOME SEQUENCING OF FAMILIAL NON-RET MEDULLARY THYROID CANCER (MTC) IDENTIFIES A NOVEL POTENTIAL DISEASE SUSCEPTIBILITY GENE.

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Introduction

Between 85% and 98% of familial MTC is caused by a germline mutations of the RET proto-oncogene. There are however, rare families and individuals with predisposition to MTC in whom no RET mutation has been identified (non-RET MTC). The British Thyroid Association (BTA) recommends the investigation of such families as identification of novel candidate genes may inform the molecular behaviour of more common, sporadic disease. Whole exome sequencing (WES) technology enables all protein coding regions of the genome to be sequenced in parallel; a novel technique in searching for predisposing genes in MTC. We present our experience and preliminary data using this novel approach.

Methods

Patients with non-RET familial and sporadic MTC were recruited through national and internationally developed collaborations. Germline and tumour DNA from three families with non-RET MTC and 63 cases of non-RET sporadic MTC were analysed. WES of affected family members was completed in three generations of the index family. Functional analysis to confirm the biological pathways underlying identified mutations is currently underway.

Results

WES identified over 20,000 mutations that were screened using an established protocol. A frameshift mutation has been identified in familial non-RET MTC within a single gene (MTC2) with familial segregation confirmed by Sanger sequencing. Further mutations have been identified in germline DNA from a patient with young onset sporadic disease and in tumour DNA extracted from patients with sporadic MTC. Preliminary in-vitro studies suggest RET protein up-regulation in MCF-7 cells transfected with the MTC2 mutant establishing a possible novel tumorigenic pathway for MTC development.

Conclusions

We have established a method of using WES in the context of MTC. Identification of a novel susceptibility gene represents a significant breakthrough in our understanding of MTC. As well as the potential for a genetic test, and as a prognostic biomarker, the on-going functional work may elucidate targets for novel therapies.
HEPARANASE IN THE DIFFERENTIATION OF MALIGNANT FROM BENIGN THYROID NEOPLASMS
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Background: The search for a specific marker that could help to distinguish between differentiated thyroid carcinoma and benign lesions remains elusive in clinical practice. Heparanase (HPSE) is an endo-beta-glucuronidase implicated in the process of tumor invasion, and the isoform heparanase-2 (HPSE2) modulates HPSE activity. The aim of this study was to evaluate the role of heparanases in the development and differential diagnosis of follicular pattern thyroid lesions.

Methods: HPSE and HPSE2 expression by quantitative RT-PCR (qRT-PCR), immunohistochemistry evaluation, western blot analysis and HPSE enzymatic activity was initially analyzed in the 27 samples obtained from prospectively selected patients (15 malignant lesions: papillary and follicular carcinomas; 12 benign lesions). Immunohistochemical analysis was followed up using 230 paraffin-embedded thyroid lesions that had been retrospectively selected.

Results: The expression of heparanase isoforms by qRT-PCR showed an increase of HPSE2 in thyroid carcinoma compared to the benign lesions (P=0.001). HPSE activity was found to be higher in the malignant neoplasms than in the benign tumors (P<0.0001). Immunofluorescence showed an increased expression of HPSE (P=0.039) and HPSE2 (P=0.008) in malignant lesions, which also presented an increased colocalization of both enzymes (P=0.003) and a decreased HPSE/HS ratio (P=0.039). On Western blot analysis, HPSE2 isoforms were detected only in malignant tumors. Both pro-enzyme and active HPSE isoforms were higher in malignant lesions whereas only the pro-enzyme was detected in the benign tumors. The immunohistochemical assay allowed us to establish a distinct pattern for malignant and benign tumors. While carcinomas showed a typical combination of positive labeling for neoplastic cells and negative immunostaining in colloid, benign tumors showed other patterns (P<0.0001 - Figure 1). The proposed diagnostic test presents sensitivity and negative predictive value of around 100%, showing itself to be an accurate test for distinguishing between malignant and benign lesions.

Conclusions: This study shows, for the first time, a distinct profile of HPSE expression in thyroid carcinoma suggesting its role in carcinogenesis. Furthermore, the pattern of HPSE2 expression was established as an accurate marker in the distinction between benign and malignant thyroid tumors with real potential application for diagnosis in clinical practice.
Figure 1. Follicular carcinoma. Different patterns of HPSE2 labeling observed in the same photomicrography: adjacent normal thyroid tissue (arrow head - HPSE2 was observed preferentially in colloidal areas) and follicular carcinoma (arrow - HPSE2 was positive in neoplastic cells and negative on colloid). Immunohistochemistry - optical microscopy, X200.
Background. Papillary thyroid carcinoma (PTC) is the most common malignant endocrine neoplasia with an evident increase in incidence. Considering that most of the thyroid lesions diagnosed is proven to be benign, most of these patients are exposed to unnecessary surgical hazards, in addition to the need of a lifelong thyroid hormone replacement therapy. The goal of this study was to identify accurate molecular diagnostic markers in PTC.

Methods. Expression profiling was carried out in 61 PTC and 13 adjacent non-neoplastic tissues (NT) using Sure Print G3 8x60K oligoarray slides (Agilent Technologies). Transcription profiling data (microarray and RNA sequencing) from 138 matched PTC and NT were obtained from available public databases (GEO and TCGA portal). A panel of 28 transcripts was further evaluated with RT-qPCR, including benign thyroid lesions (BTL) and a diagnostic algorithm was trained (86 PTC, 23 NT and 8 BTL) and validated (120 PTC, 10 NT and 19 BTL).

Results. Microarray analysis in combination with meta-analysis identified 589 genes differentially expressed in PTC compared to NT, where GABRB2 was ranked as the most up-regulated gene in PTC. By RT-qPCR, all 28 transcripts were confirmed as involved in PTC. Combined expression of CLDN10, HMGA2 and LAMB3 was able to discriminate all samples in the training set. This algorithm achieved an area under the curve (AUC) of 0.99 with 97.5% of sensitivity and 90.3% of negative predictive value (NPV) in the validation set. Cases with higher algorithm scores were associated with lymph node involvement.

Conclusions: This study revealed candidate markers able to discriminate PTC from normal/benign thyroid tissues.
IN SILICO ANALYSIS OF RET MUTATIONS IN MEDULLARY THYROID CANCER: FROM THE COMPUTER TO THE BEDSIDE

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Background:

Multiple endocrine neoplasia (MEN) type 2 is a rare genetic disorder, in which nearly all patients develop medullary thyroid cancer (MTC). MEN2 is an autosomal dominant condition that may affect many members of a family over several generations. Once a proband is diagnosed with MEN2, the current recommendation is to screen family members and perform a prophylactic thyroidectomy in the kindred at risk of developing MTC. Over the past two decades, analysis of genotype-phenotype relationships has shown a distinct relationship between a given mutation in the RET gene and MEN2 disease presentation. While the significance of certain mutations is well documented, there remain some mutations whose effect on RET activation is still unknown. This data culminated in the American Thyroid Association (ATA) guidelines for prophylactic thyroidectomy in MEN2 based on three classes of mutations: surgery before 5 (Level C), before 5 but possibly delayed (Level B), and before 10 years of age (Level A).

Methods:

An in silico analysis of known RET gene mutations was performed using Align GVGD software (http://www.agvgd.iarc.fr). There are two components to the Align GVGD algorithm: Grantham Variation (GV) and Grantham Deviation (GD). GV is a measure of the variation in the amino acid sequence between other species to determine if a particular region of the protein is highly conserved, and therefore, critical to proper function. In this study, we aligned the sequences of human, rhesus monkey, dog, cattle, mouse, rat, chicken, and zebrafish RET genes. GD is a measure of the biochemical difference between mutant and wild-type protein, and the resultant alteration is structure by biophysical modeling. The combination of GV and GD yields a prediction in the amount of structural difference observed with a given mutation, from no difference (0) to maximum difference (65). We examined all mutations currently listed in the ATA mutation guidelines for prophylactic thyroidectomy.

Results:

We found a statistically significant difference in the overall GVGD score between ATA groups A, B, and C. The RET mutations associated with a more aggressive clinical phenotype generally had a higher GVGD score. The mean GVGD scores of ATA groups A-C were 35 (n=17), 55 (n=27), and 65 (n=6) respectively. All mutations in ATA group C had the highest GVGD score (65). An independent t-test comparison of the mean of ATA group A to B was p=0.04; ATA group B to C was p=0.01. Additionally, in mutations known to have a higher predisposition to primary hyperparathyroidism, 24/26 (92%) had the highest GVGD score.

Conclusions:

We have shown that Align GVGD was able to accurately distinguish ATA groups from one another on the basis of the mutation alone. The use of computer-based protein modeling to predict clinical outcomes for rare, genetic diseases, such as the timing of prophylactic thyroidectomy in MEN2A, may add crucial
evidence to aid in decision-making. Furthermore, if a previously unknown mutation (non-ATA classified) is discovered in a patient, in silico analysis could determine the potential aggressiveness and predict the ideal age for prophylactic thyroidectomy.
THE POTENTIAL UTILITY OF MOLECULAR ANALYSIS OF THYROID NEEDLE ASPIRATION CYTOPATHOLOGY SPECIMENS IN A REFERRAL UNIVERSITY CENTER: ANALYSIS OF 2216 THYROID NEEDLE ASPIRATIONS

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Background & Objective: Over the last 10 years there has been a proliferation of molecular analyses utilized to refine the diagnostic results of thyroid fine-needle aspiration cytopathology. Several of these are now offered as commercially available analyses, and a number are being utilized noncommercially. This study was designed to determine the potential utility of the commercially available Afirma gene-expression classifier (Veracyte Inc., San Francisco, CA), and a panel of molecular markers similar to those available through the miRInform® product (Asura gen, Austin, Texas), in the evaluation of thyroid nodules at a high volume university center.

Methods: A review of all thyroid fine-needle aspiration (FNA) diagnoses made by University of Iowa cytopathologist from January 1, 2010 to January 1, 2013 was conducted. Nodules were characterized based on the Bethesda classification scheme. Within the group of indeterminate nodules, available surgical histopathology results were correlated with the cytopathology results. Based on published sensitivity and specificity data for the Afirma gene-expression classifier (GEC), negative and positive predictive values for this test were calculated using the prevalence of cancer within our study population of indeterminate nodules. Based on published sensitivity and specificity data for a panel of molecular analyses including BRAF, RAS, RET/PTC and PAX8/PPARgamma, descriptive statistical predictions for our patient population were performed.

Results: A total of 2,216 thyroid FNAs were performed during the study period. Of these FNAs the cytopathologic diagnoses were: 1688 (76.2%) benign, 119 (5.4%) non-diagnostic, 145 (6.5%) malignant, and 264 (11.9%) indeterminate. Within the indeterminate group there were 44 (2%) follicular lesions of undetermined significance (FLUS), 189 (8.5%) follicular neoplasm or suspicious for follicular neoplasm (FNs) and 31 (1.4%) suspicious for malignancy (SM). Surgical histopathology was available for 173 (65.5%) of the indeterminate nodules. The prevalence of cancer within the indeterminate subgroups was: FLUS 39%, FNs 27%, SM 92%. The overall prevalence of malignancy within the indeterminate group was 37.6%. Within our patient population the negative predictive values for the Afirma GEC were: FLUS 89%, FNs 93%, SM 43%. Use of the molecular markers BRAF V600E, NRAS codon 61, HRAS codon 61, KRAS codons 12/13 and the mutations RET/PTC1 &3 and PAX8/PPARgamma in the preoperative evaluation of the FLUS (sensitivity 63%) and FNs (sensitivity 57%) would have potentially altered the initial surgical management of 24 (13.8%) of the 173 patients with indeterminate nodules that underwent surgery.

Conclusions: The way in which cytopathologic diagnoses are characterized and the prevalence of cancer within a particular institutional or referral population will influence the potential utility of molecular analyses of thyroid FNA specimens. Based on the prevalence of cancer in our patient population, the Afirma GEC was found to have potential utility only in the FNs population. This represented, at most, only 8.5% of all our thyroid FNA specimens. Selective use of a panel of mutations does offer the potential to refine the initial diagnosis and surgical management plan in some patients diagnosed with a FLUS or FNs.
WNT INHIBITORY FACTOR 1 REDUCES CELL PROLIFERATION, SELF-RENEWAL AND EPITHELIAL-MESENCHYMAL TRANSITION MARKERS IN MALIGNANT SALIVARY GLAND CELLS

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Background: Wnt/β-catenin signaling pathway regulates various key processes such as embryonic development, stem cell self-renewal, adult tissue homeostasis and oncogenesis. Studies have shown that downregulation of Wnt inhibitory factor 1 (WIF1) and hyperactivation of Wnt pathway lead to many human cancers. However, the status of WIF1 in human salivary gland cancer is not known.

Aims: 1) To determine the level of WIF1 protein expression in human salivary gland tumors and to delineate the mechanisms that contribute to WIF1 downregulation in salivary gland cancer. 2) To investigate the effects of WIF1 on human salivary gland cancer cell growth, cancer stemness, epithelial-mesenchymal transition (EMT) markers and senescence.

Methods: WIF1 protein expression was determined in a large series of benign and malignant salivary gland tumors by immunohistochemistry. To determine the mechanism of WIF1 downregulation, we performed methylation-specific PCR in WIF1 promoter and loss of heterozygosity (LOH) studies in human normal and malignant salivary gland samples. To delineate the tumor suppressive effects of WIF1 on malignant salivary gland cells, carcinoma ex-pleomorphic adenoma (CaExPA79) cells were transiently transfected with empty vector or pCI blast-WIF1. Cell proliferation, self-renewal and EMT markers were studied by hexosaminidase assay, colony formation assay and real-time RT-PCR analysis, respectively. The effects of WIF1 on cancer stem cells and senescence were determined by ALDEFLUOR assay and senescence associated β-galactosidase staining, respectively.

Results: In normal salivary gland, WIF1 was detected as an abundant cytoplasmic brown granular staining and highly expressed. In contrast, WIF1 protein expression was down-regulated in benign tumors and undetectable in most of the malignant samples. Methylation-specific PCR and LOH analyses showed that promoter hypermethylation and loss of genetic material contribute to downregulation of WIF in human salivary gland tumors. WIF1 decreased the salivary gland cancer cell proliferation and spheroid formation. Importantly, WIF1 significantly reduced the pluripotency and self-renewal markers (c-MYC, OCT4, c-KIT, MYB, WNT3A and TCF4) in malignant salivary gland cells. Furthermore, WIF1 reduced the expression of the markers of EMT as observed by downregulation of BMI1, ZEB1 and ZEB2, with upregulation of E-cadherin. Remarkably, WIF1 decreased the number of cancer stem cells, while inducing a more differentiated phenotype and increasing cellular senescence.

Conclusions: Our findings demonstrate for the first time that WIF1 downregulation is a frequent event in human malignant salivary gland. Promoter hypermethylation and loss of genetic material appear to be the main mechanisms involved in WIF1 downregulation. WIF1 inhibits the growth of malignant salivary gland cells by decreasing cell proliferation, cancer stem cells, self-renewal and EMT markers, and increasing cellular senescence. Our study emphasizes the therapeutic value of WIF1 in the treatment of salivary gland cancer and highlights the importance of this Wnt antagonist in the development of molecular therapies to cure human cancer.
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GENETIC AND EXPRESSION ANALYSIS OF HER2 IN SALIVARY DUCT CARCINOMA

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Background. Salivary duct carcinoma (SDC) has an extremely poor prognosis in comparison with other salivary gland carcinomas. Recently, molecular biological analysis has been widely performed in cases of head and neck carcinoma for the development of molecular-targeted therapies. In this study, we analyzed the expression of HER2 protein, which has been recognized as a potential target for molecular-targeted therapy.

Methods. This study comprised 20 patients with SDC of the major salivary glands who were treated at Hokkaido University Hospital, Sapporo, Japan, between January 1990 and March 2010. HER2 protein expression and gene amplification were detected by immunohistochemical analysis and fluorescence in situ hybridization. Furthermore, to examine HER2 receptor activity, the downstream signaling of HER2 in cells was examined by immunohistochemical analysis.

Results. HER2 was overexpressed in 15 cases (75%); 5 cases showed a score of 2+ and 10 cases showed a score of 3+. Amplification of the HER2 gene was found in 11 tumors (55%); with 9 revealing a score of 3+ and 2 revealing a score of 1+. As tumors with an expression score of 3+, or 2+ with gene amplification were regarded as positive for HER2, 10 cases were judged to be HER2-positive. HER2-positive tumors have no correlation with gender, age, T and N classification, or perineural invasion. However, HER2-positive tumors were statistically more often generated from pleomorphic adenoma than de novo. (P = 0.005) The survival rate was analyzed for 19 patients who underwent definitive therapy. The 5-year overall survival rate was 35.6% for patients with a HER2-positive tumor (n=10) and 66.7% for those with a HER2-negative tumor (n=9).

Conclusions. Our study indicated that overexpression of HER2 is likely to be a poor prognostic factor for patients with SDC. HER2 is one possible target for therapy for SDC; however, further clinical studies are required to determine the optimum regimen for combination therapy including trastuzumab and the selection of eligible patients.
BRAF MUTATION IS NOT ASSOCIATED WITH INCREASED RECURRENCE OR MORTALITY IN PAPILLARY THYROID CARCINOMA

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Background: BRAF-mutated papillary thyroid carcinomas (PTC) are associated with a high incidence of lymph node metastasis and a suggested relationship with increased mortality. We sought to investigate this relationship in a high-risk population.

Methods: Consecutive, prospectively enrolled patients with PTC undergoing comprehensive surgical management for radiographic and/or clinical evidence of persistent/recurrent PTC underwent genomic DNA analysis of their tumors using high throughput mass array and next generation sequencing. Two-tailed Fisher exact tests were performed to analyze for associations between BRAF and further recurrences, as well as age, gender, and other metrics of the cohort. Overall survival (OS), disease-free survival (DFS), and disease-free interval to recurrence was evaluated with respect to BRAF mutation by log rank test analysis of Kaplan-Meier plots.

Results: Genomic data was obtained from 257 consecutive patients with PTC undergoing surgery for recurrent disease. Of these, 148 (57.6%) were females and 108 (42.4%) were males. About half (49.8%) were less than age 45 and half (50.2%) were 45 or older at the time of surgery. BRAF mutation was identified in 236 patients (91.8%). No association was identified between BRAF mutational expression and a further PTC recurrence. Of the 236 patients who expressed BRAF, 70 (29.8%) had second PTC recurrences while 8 of the 21 patients (38.1%) not found to express BRAF did (2-tailed Fisher exact test p = 0.46). No association was noted between BRAF mutation and either gender (p = 0.25) or age (p = 0.12). BRAF expression did not impact OS (p=0.59), DFS (p=0.88), or disease-free interval to recurrence (p=0.33). Subset analysis in patients over 50 years of age also showed no association between BRAF expression and survival. Women were significantly more likely to be of younger age at first recurrence (mean age of recurrence for women, 42.9, mean age of recurrence for men, 51.7, p=0.00005).

Conclusions: BRAF mutation was not found to be associated with further PTC recurrence in this cohort of consecutive patients undergoing surgical management for recurrent/persistent PTC. BRAF expression had no impact on either survival metrics or interval to recurrence, including in those patients over 50 years of age. These data support the hypothesis that BRAF does not have a significant impact on risk of recurrence, regardless of age or gender, nor does it significantly impact survival metrics.