Meta-Analysis and Tissue Microarray Analysis Identifies Promising Biomarkers for Thyroid Cancer

Obi L Griffith[1,2], Adrienne Melck[3], Allen Gown[4], Sam M Wiseman[3,5], and Steven JM Jones[1,2]

1. Canada's Michael Smith Genome Sciences Centre, British Columbia Cancer Agency; 2. Department of Medical Genetics, University of British Columbia; 3. Department of Surgery, University of British Columbia; 4. Department of Pathology, University of British Columbia; 5. Genetic Pathology Evaluation Center, Prostate Research Center of Vancouver General Hospital & British Columbia Cancer Agency

British Columbia Cancer Agency
Vancouver, British Columbia, Canada

Canada's Michael Smith Genome Sciences Centre
www.bcgsc.ca

1. Abstract

Objective and Design: An estimated 4-7% of the population will develop a clinically significant thyroid nodule during their lifetime. In many cases pre-operative diagnoses by needle biopsy are inconclusive. Thus, there is a clear need for improved diagnostic tests to distinguish malignant from benign thyroid tumors. The recent development of high throughput molecular analytic techniques should allow the rapid evaluation of new diagnostic markers. However, researchers are faced with an overwhelming number of potential markers from numerous thyroid cancer expression profiling studies. To address this we performed a systematic identification of potential thyroid cancer biomarkers from published studies by meta-analysis followed by tissue microarray analysis (TMA).

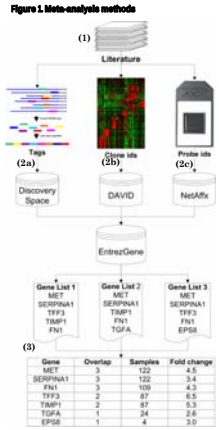
Materials & Methods: A total of 21 thyroid expression studies were identified from the literature. A heuristic system was devised to identify the most promising markers, taking into consideration the number of studies reporting the potential marker, sample sizes, and fold-changes. TMAs consisting of 100 benign thyroid lesions and 105 malignant thyroid lesions were stained for 56 markers. However, only a few markers from the meta-analysis have been processed so far. Significant associations between marker staining and diagnosis (benign versus malignant) were determined using contingency table statistics and Mann-Whitney U-test (MU) test (where appropriate). The samples and markers were clustered using a simple hierarchical clustering algorithm and evaluated for their utility in classification (benign vs. malignant) using the Random Forests (RF) classifier algorithm.

Results: A total of 755 genes were reported from 21 comparisons and of these, 107 genes were reported more than once with a consistent fold-change direction. This result was highly significant (p<0.0001). Comparison to a subset analysis of microarrays re-analyzed directly from raw image files found some differences but a highly significant concordance with our method (p-value = 6.47E-68). In total, 34 of the 56 markers tested on TMA were found to be significantly associated with diagnosis. Of these, 7 markers were down-regulated (malignant vs. benign) and 27 up-regulated. The RF algorithm was able to achieve a good classification of patients into their correct diagnostic group using marker score with a sensitivity of 88.5%, specificity of 94% and overall error rate of only 8.7%.

Conclusion: Bioinformatics meta-analysis and tissue microarray analysis represents a powerful approach to identifying new thyroid cancer biomarkers. Additional candidates from the meta-analysis should help to develop a panel of markers with sufficient sensitivity and specificity for the diagnosis of thyroid tumors in a clinical setting.

2. Methods

Figure 1. Meta-analysis methods

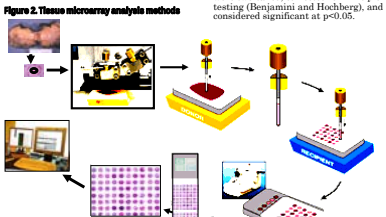


Gene	Overlap	Samples	Fold change
MET	3	122	4.5
SERPINA1	3	122	3.4
FN1	3	109	4.3
TFF3	2	87	6.5
TIMP1	2	87	5.3
TGFA	1	24	2.6
EPS8	1	4	3.0

Fig 1: (1) Lists of differentially expressed genes were collected and curated from published studies. Each study consists of one or more comparisons between pairs of conditions (e.g. PTC vs. norm). The following information was recorded wherever possible: Unique identifier (probe, tag, accession); gene description; gene symbol; comparison conditions; sample numbers for each condition; fold change; direction of change. (2) SAGE tags, cDNA clone ids and Affymetrix probe ids were mapped to Entrez Gene using: (a) DiscoverySpace; (b) DAVID; and (c) Affymetrix annotation files. (3) Genes were ranked according to several criteria in the following order of importance: (i) number of comparisons in agreement (ie. listing the same gene as differentially expressed and with a consistent direction of change); (ii) total number of samples for comparisons in agreement; and (iii) average fold change reported for comparisons in agreement.

Fig 2: Archived thyroid cancer specimens were reviewed and selected for TMA construction. Cores were taken from each marked tumor and transferred to defined coordinates in the recipient TMA block. Blocks were cut into serial sections and transferred to slides for IHC staining. Pathologists blinded to the clinical information determined semi-quantitative marker expression scores. Scores were entered into a spreadsheet, processed by custom TMA-deconvoluter software, and finally transferred into a master study database with all clinical and pathologic patient data. Significant associations between marker staining and diagnosis were determined using contingency table statistics and Mann-Whitney U-test (MU) where appropriate. The markers were further analyzed using hierarchical clustering and Random Forests classifier algorithms. P-values were two-tailed, corrected for multiple testing (Benjamini and Hochberg), and considered significant at p<0.05.

Figure 2. Tissue microarray analysis methods



3. Existing thyroid cancer expression data

Table 1. Thyroid cancer profiling studies included in analysis

Study	Platform	Genes/ features	Comparison		Up-/down
			Condition 1 (No. samples)	Condition 2 (No. samples)	
Chen *et al.* 2001	Atlas cDNA (Clontech)	588	M (1)	FTC (1)	18/40
Arnaldi *et al.* 2005	Custom cDNA	1807	FCL(1)	Norm (1)	9/20
			PCL(1)	Norm (1)	1/8
			UCL(1)	Norm (1)	1/7
			FCL(1), PCL(1), UCL(1)	Norm (1)	3/6
Huang *et al.* 2001	Affymetrix HG-U95A	12558	PTC (8)	Norm (8)	24/27
Aldred *et al.* 2004	Affymetrix HG-U95A	12558	FTC (9)	PTC(6), Norm(13)	142/0
			PTC (6)	PTC(9), Norm(13)	0/68
Cerutti *et al.* 2004	SAGE	N/A	FA(1)	FTC(1), Norm(1)	5/0
			FTC(1)	FA(1), Norm(1)	12/0
Eszlinger *et al.* 2001	Atlas cDNA (Clontech)	588	AFTN(3), CTN(3)	Norm(6)	0/16
Finley *et al.* 2004*	Affymetrix HG-U95A	12558	PTC(7), FVPTC(7)	FA(14), HN(7)	48/85
Zou *et al.* 2004	Atlas cancer array	1176	MACL(1)	ACL(1)	43/21
Weber *et al.* 2005	Affymetrix HG-U133A	22283	FA(12)	FTC(12)	12/84
Hawthorne *et al.* 2004	Affymetrix HG-U95A	12558	GT(6)	Norm(6)	1/7
			PTC(8)	GT(6)	10/28
			PTC(8)	Norm(8)	4/4
Onda *et al.* 2004	Amersham custom cDNA	27648	ACL(11), ATC(10)	Norm(10)	31/56
Wasenius *et al.* 2003	Atlas cancer cDNA	1176	PTC(18)	Norm(3)	12/9
Barden *et al.* 2003	Affymetrix HG-U95A	12558	PTC(9)	FA(10)	59/45
Yano *et al.* 2004	Amersham custom cDNA	3968	PTC(7)	Norm(7)	54/0
Chevillard *et al.* 2004	custom cDNA	5760	FTC(3)	FA(4)	12/31
			FVPTC(3)	PTC(2)	12/16
Mazzanti *et al.* 2004	Hs-UniGem2 cDNA	10000	PTC(17), FVPTC(15)	FA(16), HN(15)	5/41
Takano *et al.* 2000	SAGE	N/A	FTC(1)	ATC(1)	3/10
			FTC(1)	FA(1)	4/1
			Norm(1)	FA(1)	6/0
			PTC(1)	ATC(1)	2/11
			PTC(1)	FA(1)	7/0
			FTC(1)	FTC(1)	2/1
Finley *et al.* 2004*	Affymetrix HG-U95A	12558	FTC(9), PTC(11), FVPTC(13)	FA(16), HN(10)	50/55
Pauws *et al.* 2004	SAGE	N/A	FVPTC(1)	Norm(1)	33/9
Jarzab *et al.* 2005	Affymetrix HG-U133A	22283	PTC(16)	Norm(16)	75/27
Giordano *et al.* 2005	Affymetrix HG-U133A	22283	PTC(51)	Norm(4)	90/151
21 studies	**10 platforms**		**34 comparisons (473 samples)**		**1785**

Table 1: A total of 34 comparisons were available from 21 studies, utilizing at least 10 different expression platforms. The numbers of 'up-/down-regulated' genes reported are for condition 1 relative to condition 2 for each comparison as provided. Only genes that could be mapped to a common identifier were used in our subsequent analysis (see methods). Several comparison groupings were analyzed but here we will only discuss the 'cancer vs. non-cancer' comparison grouping. There were 21 comparisons (in blue) which compared some kind of cancer tissue with some kind of non-cancer tissue (normal or benign). *Two studies by Finley *et al* had significant overlap in the samples analyzed. Only the larger study was included to avoid spurious overlaps.

4. Meta-analysis results

Figure 3. Gene overlap for cancer vs. non-cancer analysis

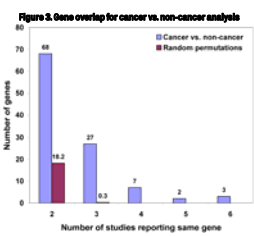


Fig. 3: a total of 755 genes were reported from 21 comparisons, and of these, 107 genes were reported more than once with consistent fold-change direction. In some cases (e.g., MET, TFF3, and SERPINA1), genes were independently reported as many as six times. The total amount of overlap observed was assessed by Monte Carlo simulation (represented by the red bars) and found to be highly significant (P<.0001; 10,000 permutations).

Table 2: Shows a partial list of genes (identified in 4 or more comparisons) from the cancer vs. non-cancer analysis. A complete table for this group and all others are available as supplementary data (www.bcgsc.ca/bioinfo/ge/thyroid/). A review of these candidates revealed both well known thyroid cancer markers as well as relatively novel or uncharacterized genes.

Table 2. Cancer versus non-cancer genes identified in 4 or more independent studies

Gene	Description	Comp's (Up/Down)	N	Fold Change
MET	met proto-oncogene (hepatocyte growth factor receptor)	6/0	202	3.03
TFF3	trefoil factor 3 (intestinal)	6/0	196	14.70
SERPINA1	serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1	6/0	192	15.84
EPS8	epidermal growth factor receptor pathway substrate 8	5/0	186	3.15
TIMP1	tissue inhibitor of metalloproteinase 1 (erythroid potentiating activity, collagenase inhibitor)	5/0	142	5.38
TGFA	transforming growth factor, alpha	4/0	165	4.64
QPCT	glutaminyl-peptide cyclotransferase (glutaminyl cyclase)	4/0	153	7.31
PROS1	protein S (alpha)	4/0	149	4.32
CRABP1	cellular retinoic acid binding protein 1	0/4	146	-11.55
FN1	fibronectin 1	4/0	128	7.68
FCGBP	Fc fragment of IgG binding protein	0/4	108	-2.41
TPO	thyroid peroxidase	0/4	91	-4.69

5. Tissue microarray analysis results

Table 3. Utility of stained markers for distinguishing benign from tumor.

Marker	Benign mean rank	Malignant mean rank	P-value	Var. Imp.
VEGF	130.3	65.3	0.0000	6.909
Galectin	59.1	139.6	0.0000	15.895
CK19	60.1	138.5	0.0000	13.942
AR	74.7	123.3	0.0000	5.048
AuroraA	68.1	123.2	0.0000	4.437
HBME	74.4	123.6	0.0000	5.309
P16	73.8	123.5	0.0000	4.174
BCL2	121.1	71.1	0.0000	2.383
CYCLIND1	67.0	115.5	0.0000	2.852
Caveolin1	77.5	119.1	0.0000	2.308
ECAD	120.2	75.9	0.0000	3.186
CYCLINE	77.1	118.0	0.0000	1.633
CR3	77.5	113.9	0.0000	1.045
Clusterin	79.6	117.0	0.0000	2.478
IGFBP5	79.0	112.2	0.0000	1.144
P21	81.0	113.4	0.0000	0.549
BetaCatenin	89.5	107.9	0.0000	0.295
IGFBP2	82.1	109.7	0.0001	1.051
Caveolin	78.8	109.0	0.0002	2.359
HER4	82.7	112.6	0.0003	1.273
TG	104.0	87.7	0.0003	1.268
CKIT	104.8	88.6	0.0004	0.810
S100	89.0	101.6	0.0004	0.230
KI67	86.9	101.6	0.0007	0.793
AuroraC	79.7	104.7	0.0015	1.059
RET	78.5	98.7	0.0017	0.554
HER3	83.1	103.5	0.0056	0.526
AMFR	87.5	105.0	0.0113	0.590
MLH1	101.5	94.4	0.0124	1.344
TTF1	87.7	102.9	0.0149	0.998
AAT (SERPINA1)	88.5	100.2	0.0194	1.268
Synetnophin	88.7	103.5	0.0267	0.649
HSP27	99.9	82.7	0.0351	1.498

Table 3: Of the 56 markers tested on tissue microarray, 33 were found to be significantly associated by MU test after multiple testing correction. Of these, 7 markers were down-regulated (in malignant compared to benign) and 26 up-regulated. To date, only 4 markers (in blue) from the meta-analysis candidates have been tested (chosen by availability, not rank) on the TMA. All four were found to be significant with three in the top 10 for diagnostic potential. A number of variables contributed to the classification performance with Gini variable importance (Var. Imp.) values ranging from 0 to ~16. Not surprisingly, the relative order of variable importance in the RF classifier had strong concordance with the measures of significance.

Figure 4. Hierarchical clustering of 10 most significant markers

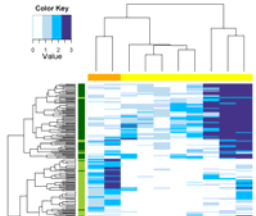


Fig. 4: All markers were submitted to the Random Forests classification algorithm with a target outcome of cancer versus benign. The Random Forests algorithm was able to achieve a good classification of patients into their correct diagnostic group using marker score with a sensitivity of 94% and overall error rate of only 8.7%. Specifically, this translates to a misclassification of only 6 out of 100 benign and 11 of 96 malignant samples. This performance is graphically illustrated by the good separation of benign samples (light green side bar) from the malignant (dark green side bar) samples in the hierarchical clustering heatmap. For illustrative purposes, only the 10 most significant markers are plotted in the heatmap. The color key represents marker scores from 0 (weak / negative) to 3 (strong / positive).

6. Conclusions

> A significant number of genes are consistently identified in the literature as differentially expressed between benign and malignant thyroid tissue samples.
> Our meta-analysis approach represents a useful method for identifying consistent gene expression markers when raw data is unavailable (as is generally the case).
> Some markers have previously undergone extensive validation while others have not yet been investigated at the protein level.
> Preliminary immunohistochemistry analysis on a TMA of over 200 thyroid samples for 56 antibodies show promising results.
> Additional candidate genes from the meta-analysis may facilitate the development of a clinically relevant diagnostic marker panel.

7. Acknowledgments and other details

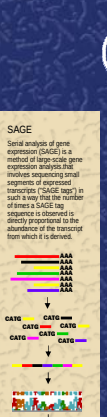
funding | Natural Sciences and Engineering Council of Canada (OG); Michael Smith Foundation for Health Research (OG, SW, and SJ); Canadian Institutes of Health Research (OG); BC Cancer Foundation

references | Griffith OL, Melck A, Jones SJM, Wiseman SM. 2006. A Meta-analysis and Meta-review of Thyroid Cancer Gene Expression Profiling Studies Identifies Important Diagnostic Biomarkers. Journal of Clinical Oncology. 24(31):5043-5051.

Abbreviations | ACL, Anaplastic thyroid cancer cell line; APTN, Autonomously functioning thyroid nodules; ATC, Anaplastic thyroid cancer; CTN, Cold thyroid nodule; DTC, Differentiated thyroid cancer; FA, Follicular adenoma; FCL, Follicular carcinoma cell line; FTC, Follicular thyroid carcinoma; FVPTC, Follicular variant papillary thyroid carcinoma; GT, Goiter; HCC, Hurthle cell carcinoma; HN, Hyperplastic nodule; M, Metastatic; MACL, Anaplastic thyroid cancer cell line with metastatic capacity; MTC, Medullary thyroid carcinoma; Norm, Normal; PCL, Papillary carcinoma cell line; PTC, Papillary thyroid carcinoma; TCVPTC, Tall-cell variant papillary thyroid carcinoma; UCL, Undifferentiated carcinoma cell line

SAGE

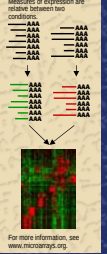
Serial analysis of gene expression (SAGE) is a method of large-scale gene expression analysis that involves sequencing small segments of expressed transcripts ("SAGE tags") in such a way that the number of times a SAGE tag sequence is observed is directly proportional to the abundance of the transcript from which it is derived.



A description of the protocol and other references can be found at www.sagenet.org.

cDNA Microarrays

cDNA Microarrays simultaneously measure expression of large numbers of genes based on hybridization to cDNAs attached to a solid surface. Measures of expression are relative between two conditions.



For more information, see www.microarrays.org.

Affy Oligo Arrays

Affymetrix oligonucleotide arrays make use of tens of thousands of carefully designed oligos to measure the expression level of thousands of genes at once. A single labeled sample is hybridized at a time and an intensity value reported. Values are the based on numerous different probes for each gene or transcript to control for non-specific binding and chip inconsistencies.

For more information, see www.affymetrix.com.