

TPMRSS2: ERG Gene Fusion Provide Insight Into The Heterogeneity Of Prostate Cancer (Approx. 40 minutes with 5 minutes for questions)
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Abstract

Prostate cancer (PCA) is a common and clinically heterogeneous disease with marked variability in progression. This talk focuses on the common *TPMRSS2-ETS* fusion event in PCA and explores the role of the translocation in disease progression. By applying a new bioinformatics approach, a common translocation in PCA was identified involving the tightly androgen regulated gene *TPMRSS2* (21q22.3) and *ETS* transcription factor family members, either *ERG*, *ETV1*, or *ETV4*. This translocation is detected in invasive PCA and in 20% of high-grade prostatic intraepithelial neoplasia (PIN). *TPMRSS2-ERG* PCA are associated with higher tumor stage and PCA specific death. *TPMRSS2* is one of the most highly androgen regulated genes. Since the original report, we have further discovered that approximately 60% of tumors with *TPMRSS2-ETS* translocations harbor deletions on chromosome 21 involving the region between *TPMRSS2* and *ERG*. The presence of translocation-associated deletions, as seen in CML, may provide important insight into the clinical and genetic heterogeneity of PCA.

Introduction

Prostate cancer (PCA) is a common and clinically heterogeneous disease with marked variability in progression. Hematological malignancies are often characterized by balanced, disease-specific chromosomal rearrangements (i.e., balanced translocations). The prototypic example is the malignant transformation of white blood cells to Chronic Myeloid Leukemia (CML) through a balanced translocation between chromosome 9 and 22 resulting in the novel tyrosine kinase fusion protein, BCR-ABL. Until recently, most solid tumors had been characterized only by non-specific chromosomal aberrations. By applying a new bioinformatics approach, our group identified a common fusion in PCA, involving the tightly androgen regulated gene *TPMRSS2* (21q22.3) and *ETS* transcription factor family members, either *ERG* (21q22.2), *ETV1* (7p21.2) or *ETV4* (17q21). This fusion is detected in invasive PCA and only in 20% of the precursor lesion high-grade prostatic intraepithelial neoplasia (PIN). A previous role of *ETS* genes in PCA progression has been suggested based on the over-expression of *ERG* and *ETV1*. New data from our group demonstrate that *TPMRSS2-ERG* fusion PCA is associated with higher tumor stage and PCA specific death in a cohort of men followed on a Watchful Waiting trial. *TPMRSS2* is one of the most highly androgen regulated genes. Since our original report, multiple independent groups have now confirmed our original observations that approximately 50-70% of PCA harbor the *TPMRSS2-ETS* gene fusion and that the most common fusion event is the *TPMRSS2-ERG* fusion. *TPMRSS2* and *ERG* genes lie in close proximity on chromosome 21. We have identified two major sources of heterogeneity among the gene fusion PCAs. First, approximately 60% of the *TPMRSS2-ERG* fusion PCA occur through the intronic deletion of a 3 Mb region between the two genes and the remaining cases presumably undergo a translocation. We have additional evidence to suggest that the presence of fusion-associated deletions, as seen in CML, may provide important insight into the clinical and genetic heterogeneity of PCA. The second source of potential heterogeneity was observed by Mike Iltmann's group and confirmed by our recent work that there are over 15 different isoforms of the *TPMRSS2-ERG* fusion transcript, suggesting that this may also help explain PCA heterogeneity.

Prostate Cancer: Predicting Risk Of Adverse Outcome

In the United States (U.S.), the prevalence of pathologic PCA is extremely high and increases with age.

One in 6 men will be diagnosed with PCA during their lifetime. PCA is a leading cause of male cancer-related death, second only to lung cancer¹⁻² and the American Cancer Society estimates that 234,460 American men will be diagnosed with PCA and approximately 30,000 will die in 2006 representing 10% of all cancer deaths in men in the United States³. Notwithstanding the sizable number of deaths, the majority of cases have indolent or slow growing tumors. Thus, the clinical dilemma is that we are over treating many men diagnosed today in the post-PSA screening era and inadequately treating those men with the potential for the most aggressive form of the disease-metastatic PCA. As demonstrated most recently by the randomized Scandinavian trial evaluating the benefit of prostatectomy over watchful waiting, surgery significantly decreased the risk of death from PCA⁴. However, this same study also suggests that 19 men need to be treated to benefit 1 man. Given the high prevalence of the disease, the aging of the population, and the morbidity of treatment, the ability to distinguish aggressive from indolent forms of PCA is therefore critical.

Current methods of stratifying tumors to predict outcome are based on clinical factors. These factors include Gleason grade (a measure of the extent of glandular differentiation), PSA level at diagnosis, clinical stage (the extent of disease beyond the prostate), and in some cases other factors such as the percent of biopsies that contain tumor cells. Nomograms and multifactorial staging schemes have been developed which aid in the prediction of biochemical relapse (PSA elevation) after local (surgical) and potentially curative therapy. While these clinical formulae are helpful, they are inaccurate in a large proportion of patients. Patients with high clinical risk factors are indeed at high risk of recurrence. The majority of patients, however, fall into low or intermediate clinical risk categories, yet these account for the majority of the recurrences observed. Most importantly, existing clinical predictors are not linked to the most meaningful clinical endpoint — PCA specific death.

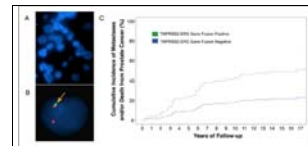
TPMRSS2-ETS Fusion in Prostate Cancer

By applying a new bioinformatics approach, our group discovered that *ERG* or *ETV1* (both members of the *ETS* family of transcription factors) were over expressed in the majority (50-70%) of PCAs and were mutually exclusive across several independent gene expression datasets, suggesting that they may be functionally redundant in PCA development⁵. Because the *ETS* family of transcription factors has previously been seen in the genomic translocation of the Ewing's Family tumors, AML, and other rare tumors, we explored the possibility that they were part of a translocation in PCA. When the *ERG* cDNA transcript was evaluated exon by exon, we determined that over expression was typically seen at the distal (3' end) but not the proximal portion (5' end). By sequencing the distal portion of the over expressed *ERG* gene, we identified that *ERG* was fused to another gene, *TPMRSS2*. While hematological malignancies are often characterized by chromosomal rearrangements (i.e., translocations), most solid tumors have a plethora of non-specific chromosomal aberrations. Thus the identification of this fusion between the prostate-specific, strongly androgen-regulated gene *TPMRSS2* (21q22.3) to *ERG* (21q22.2) or *ETV1* (7p21.2) was a surprising discovery. Using other methods to validate these findings (i.e., RT-PCR or fluorescence in situ hybridization (FISH)) in human PCA samples, we determined that the *TPMRSS2/ETS* fusion is seen in approximately 50-70% of all cases examined. Because *TPMRSS2* is regulated by androgens, we attempted a series of cell line experiments to demonstrate that exposure to androgen would specifically regulate the fused *ETS* family member. We observed that in the PCA cell line VCaP, with the *TPMRSS2-ERG* fusion exposure to a dose of synthetic androgen specifically increased *ERG* expression. In the LNCaP PCA cell lines without an *ERG* fusion, exposure to synthetic androgen did not alter *ERG* expression. Multiple groups from around the world have confirmed the finding that the *TPMRSS2-ETS* fusion in PCA is a common event¹⁻³.

TPMRSS2-ERG gene fusion PCAs are significantly associated with a higher risk of disease progression. We have recently demonstrated PCA specific death or development of metastases is associated with *TPMRSS2-ERG* fusion (Figure 1)⁴.

This study reports on a population-based cohort of men with localized prostate cancers followed by expectant (watchful waiting) therapy with 15%(17/111) *TPMRSS2-ERG* fusion. We identified a

statistically significant association between *TPMRSS2-ERG* fusion and PCA specific death (cumulative incidence ratio = 2.7, *P* < 0.01, 95% confidence interval=1.3-5.8). These data suggest that *TPMRSS2-ERG* fusion PCA may have a more aggressive phenotype, possibly mediated through increased *ERG* expression. This study was too small to determine if gene fusion through deletion had a different level of risk than fusion through translocation.



Detection of Intronic Deletions on Chromosome 21 between *TPMRSS2* and *ERG*: Since the original submission, we have now published data (Perner et al., Cancer Res 2006) describing the intronic deletions on chromosome 21 that may also suggest another possible mechanism for PCA disease progression¹⁵.
By interrogating 30 PCA samples, including cell lines, xenografts and hormone naïve and hormone refractory metastatic PCA samples, we identified genomic loss between *ERG* and *TPMRSS2* on chromosome 21q23 (Figure 2A-C). The rearrangement status for *TPMRSS2-ERG* and *TPMRSS2-ETV1* was determined for these 30 PCA by FISH and/or qPCR (Figure 2A, gray and light blue bar). None of the samples tested demonstrated a *TPMRSS2-ETV1* rearrangement. Discrete genomic loss was observed in *TPMRSS2-ERG* rearrangement positive samples involving an area between *TPMRSS2* and the *ERG* loci for LuCaP 49, LuCaP 93, ULM LN 13, MET6-9, MET18-4, MET24-26, and MET28-27. The extent of these discrete deletions was heterogeneous. More extensive genomic loss on chromosome 21 including the area between *TPMRSS2* and the *ERG* loci was observed in LuCaP 35, LuCaP 86.2, LuCaP 92.1, and MET3-81. For a subset of samples 45% (5 out of 11) the deletion occurs in proximity of *ERG* intron 3. For a majority of samples 64% (7 out of 11) the deletion ends in proximity of the SNP located on *TPMRSS2* (the next SNP in the telomeric direction is about 100k bp distant). Interestingly, for *TPMRSS2-ERG* rearrangement positive tumors, 71% (5 of 7) hormone refractory PCA demonstrate a deletion between *TPMRSS2* and the *ERG* loci whereas deletion was only identified in 25% (1 of 4) hormone naïve metastatic PCA samples (ULM LN 13). There is significant homogeneity for the deletion borders with two distinct sub-classes, distinguished by the start point of the deletion—either at 38.765 Mb or 38.911 Mb. None of the standard PCA cell lines (PC-3, LNCaP, DU-145, or CWR22 (22Rv1)) demonstrated the *TPMRSS2-ERG* or *TPMRSS2-ETV1* fusion.

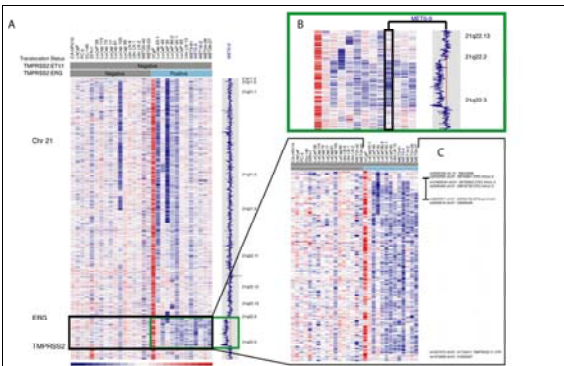
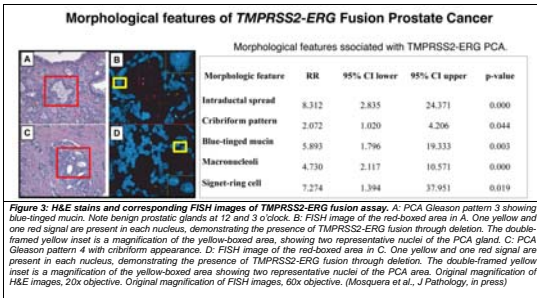


Figure 2. A-C: Genomic deletions on chromosome 21 between *ERG* and *TPMS2*. Interrogating high density 100K SNP arrays (~110,000 loci on the genome) on a panel of 30 PCA samples, we observed a commonly deleted area on chromosome 21q22.2-22.3, spanning the region between *ERG* and *TPMS2*. A. Samples, including 6 cell lines, 13 xenografts and 11 metastatic PCA samples, were characterized for *TPMRSS2-ERG* and *TPMRSS2-ETV1* status (gray bars for negative and blue bar for positive status), by qPCR and/or by FISH. B. Magnification of the green framed box in A. Signal intensity on the right side is proportional to copy number intensity of a hormone refractory metastatic PCA sample (MET6-9). Interestingly, for *TPMRSS2-ERG* rearrangement positive tumors, 71% (5 of 7) of hormone refractory PCA demonstrate a deletion between *TPMRSS2* and the *ERG* loci whereas deletion was only identified in 1 of 4 hormone naïve metastatic PCA samples (ULM LN 13). C. Magnification of the black framed box in B. SNP data include 15 loci along *ERG*, distributed from the gene promoter to exon 5 and 1 SNP on the 3' UTR of *TPMRSS2*. There is significant homogeneity for the deletion borders with two sub-classes, distinguished by the start point of the deletion—either 38.765 Mb or 38.911 Mb.

The presence of distinct morphologic phenotype associated with the *TPMRSS2-ERG* fusion PCA.

We recently explored gene fusion status of 253 PCA samples by FISH and significant associations with common morphological features¹⁶. After logistic regression analysis five morphological features were independently associated with positive *TPMRSS2-ERG* fusion status: blue-tinged mucin, cribriform pattern, macronucleoli, intraductal tumor spread, and signet-ring cell-like features (Figure 3, table). In detail, 85% (n=23/27) of cases with blue-tinged mucin (Figure 3A and B), 68% (n=50/74) of cases with cribriform pattern (Figure 3C and D), 78% (n=39/50) of cases with macronucleoli, 88% (n=38/43) of cases with intraductal tumor spread, and 82% (n=9/11) of cases with signet-ring cell-like features were *TPMRSS2-ERG* fusion positive. These five morphologic features comprise the best model that predicts a positive *TPMRSS2-ERG* fusion status. In addition to some potentially useful clinical implications for diagnosis and risk assessment, the association between phenotype and *TPMRSS2-ERG* fusion suggest

that there are molecular alterations associated with gene fusion PCA. This is the first time that a specific somatic alteration has been tied to phenotypic changes in PCA.

**Summary**

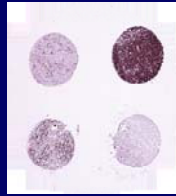
TPMRSS2-ETS gene fusion PCA is common and occurs early in the development of invasive adenocarcinoma. Considering the high incidence of PCA and the high frequency of the fusion, *TPMRSS2-ERG* is the most frequent gene rearrangement described, although the exact frequency needs to be determined in population-based studies. The clinical utility as a tissue biomarker and diagnostic tool is promising given the high specificity. Emerging data suggest that gene fusion TMA demonstrate a distinct morphologic and clinical course thus support its use as a prognostic biomarker and the fusion as an important candidate for the development of clinical therapy.

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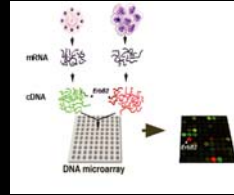
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Using sarcoma expression profiles to study tumor stroma



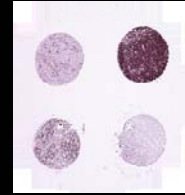
Matt van de Rijn, Stanford University

Gene arrays



Gene expression profiling:
Determines level of mRNA
for >30K genes in single
experiment

Tissue arrays



Verify and extend findings
to hundreds of cases on
tissue microarray by IHC
or ISH

Identify new marker for a sarcoma type on limited number of cases

Prognostic models in breast ca

Discovery based

Basal cell subtype
(Perou, Sorlie)

Direct fitting survival data

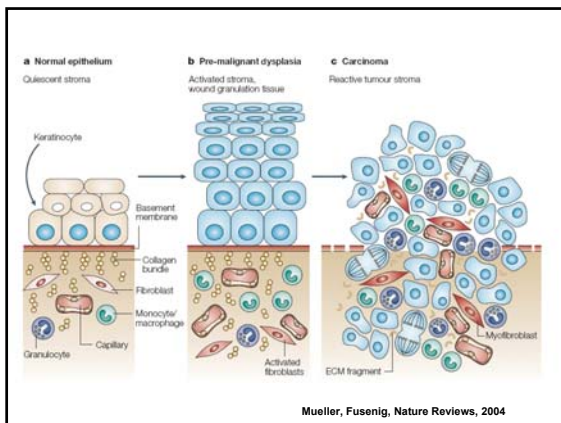
70 genes set
(van 't Veer, van de Vijver)

Hypothesis driven

Core serum response
(Chang)
DTF/SFT derived geneset
CSF1 derived geneset
(West)

Tumor-stroma interactions have biological significance

How can we discover new
stromal genes that play a role
in these interactions?



Problem:

- It is difficult to study bland spindle cells in connective tissue.
 - Morphologically highly similar
 - Few markers exist
 - Few known subtypes
 - Difficult to purify
- Opportunity: Soft tissue tumors (100 different types) are transformed normal connective tissue cells (leiomyosarcoma, liposarcoma, etc.)

HYPOTHESIS

- 1 Two cytologically similar “fibroblastic” tumors – Solitary Fibrous Tumor (SFT) and Desmoid Type Fibromatosis (DTF)- are derived from distinct connective tissue cells
 - Cell types that we currently don't recognize.

HYPOTHESIS

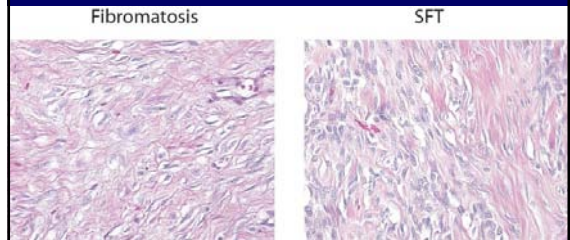
- 1 Two cytologically similar “fibroblastic” tumors – Solitary Fibrous Tumor (SFT) and Desmoid Type Fibromatosis (DTF)- are derived from distinct connective tissue cells
 - Can we use soft tissue tumors to discover new stromal cell markers similar to the use of lymphomas in the discovery of lymphocyte differentiation markers?

HYPOTHESIS

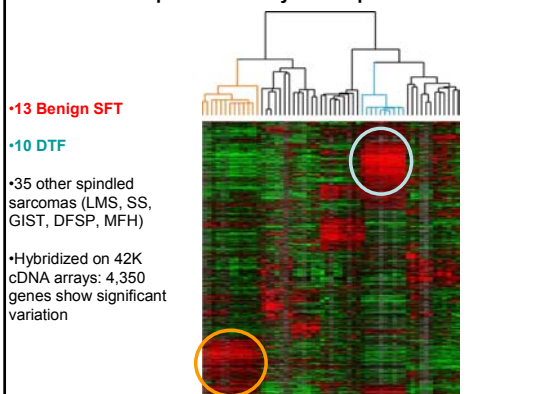
- 1 Two similar “fibroblastic” tumors – SFT and DTF- are derived from distinct stromal cells.
- 2 Breast carcinoma has an abnormal stromal component. Markers specific for DTF or SFT may help distinguish patterns of stromal response in breast carcinoma.

1st study

DTF and SFT consist of cells that look cytologically similar
(but the tumors have different growth patterns and clinical behavior)



Expression Analysis of Spindled Sarcomas



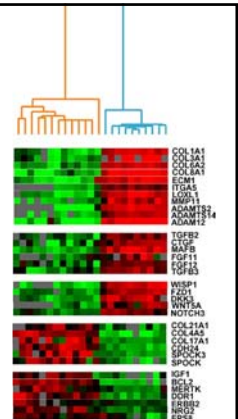
DTF vs. SFT

The genes that distinguish DTF and SFT suggest different functions for the cells from which they are derived.

Scar

Pro-fibrotic

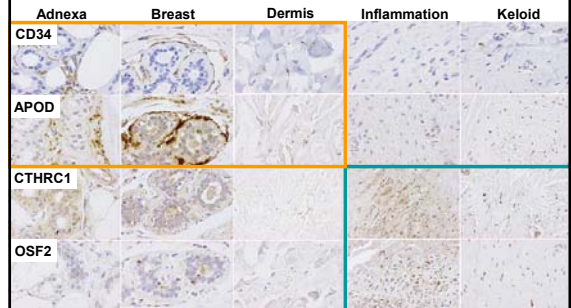
Basement membrane



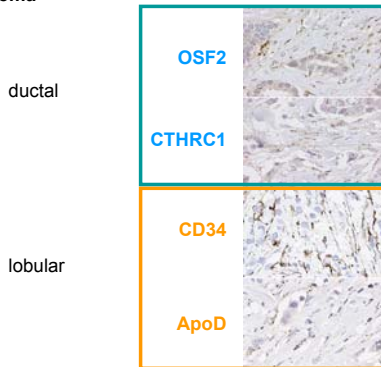
Confirmation of gene array data by IHC and ISH on
SFT and DTF

	SFT	DTF
IGF1	+	-
APOD	+	-
CD34	+	-
STAT6	+	-
K0182	+	-
CTHRC1	-	+
OSF2	-	+

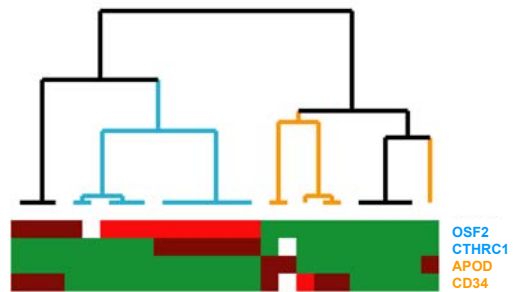
Fibromatosis and SFT markers are differentially expressed in non-neoplastic tissues



DTF and SFT markers are differentially expressed in breast carcinoma



Tissue microarray data - breast carcinoma, 24 cases

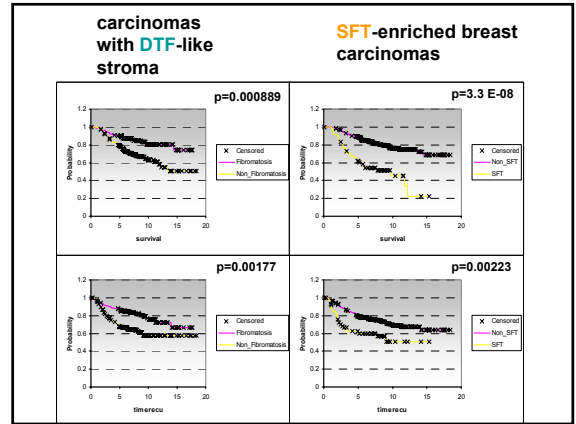
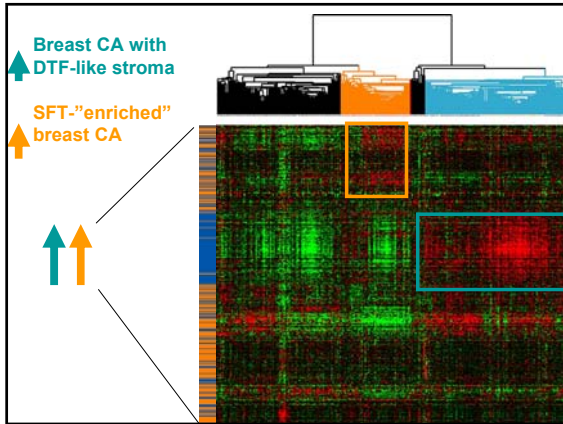


van de Vijver et al. NEJM, 2002

- 295 breast carcinoma specimens from the Dutch Cancer Institute
- mRNA isolated from whole specimens (containing tumor cells and stroma)
- 25K spot arrays
- Median follow-up - 7.8 years

van de Vijver et al. NEJM, 2002

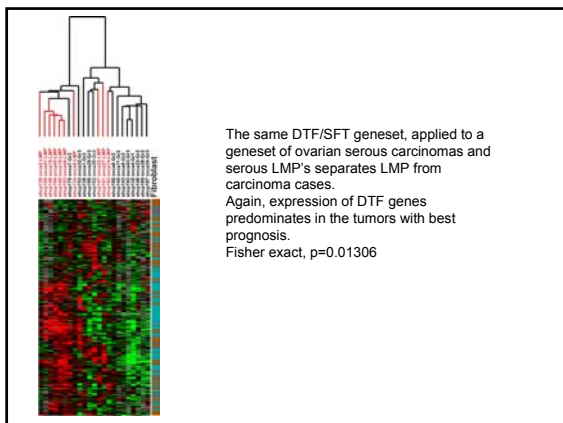
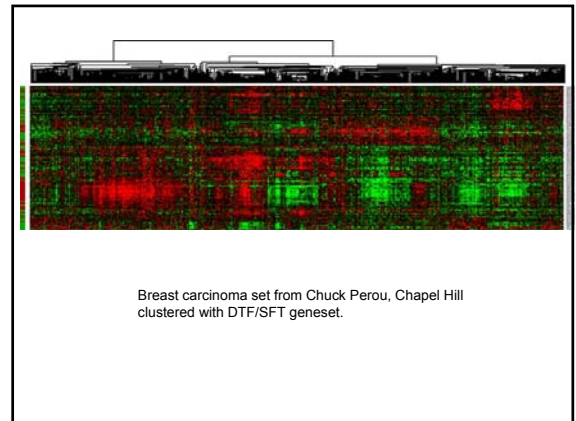
- 295 breast carcinoma specimens from the Dutch Cancer Institute
- WE SELECTED THE 470 "TOP" GENES THAT DISTINGUISH SFT AND DTF FROM OUR DATASET AND THAT WERE ALSO PRESENT ON THE DUTCH GENE ARRAYS
- WE USED THE DATA FOR THESE 470 GENES IN THE DUTCH DATASET TO CLUSTER THEIR BREAST CA CASES



Conclusions, 1st study

1. Analysis of soft tissue tumor gene expression can give insight into normal connective tissue cell subtypes
2. These soft tissue tumor genes are differentially expressed in breast cancer stroma and ovarian tumor stroma
3. (This expression pattern correlates with outcome in breast carcinoma)
4. Gene expression profiling of soft tissue tumors can be used as GENOME-WIDE SEARCH TOOL to study tumor-stroma interactions
5. The geneset derived from these STT is different from the "wound-healing"/ serum response geneset.

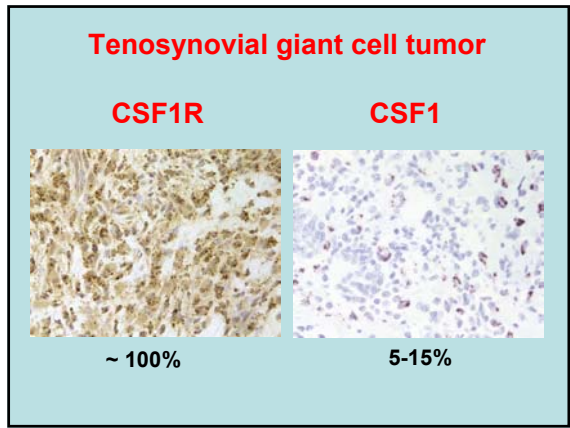
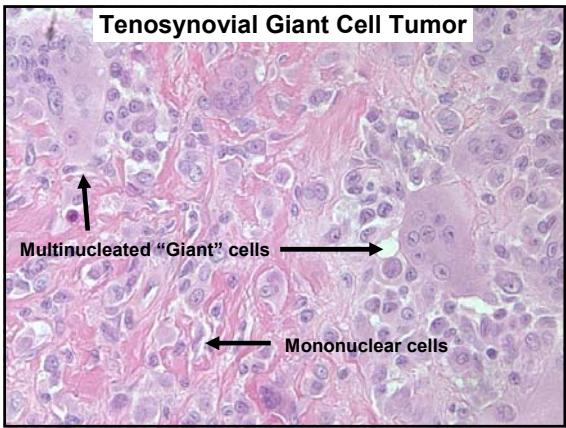
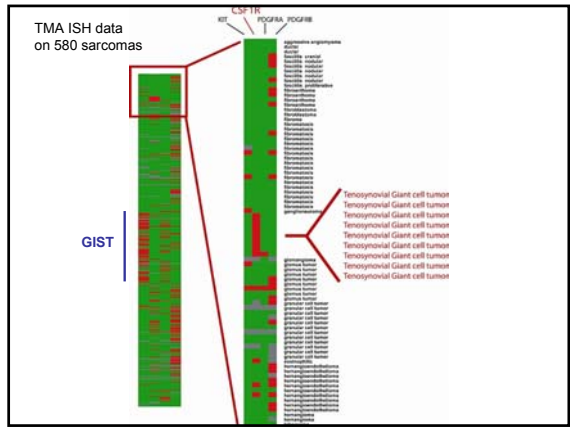
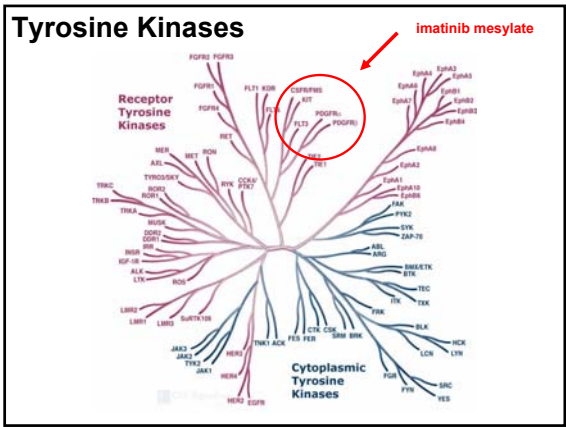
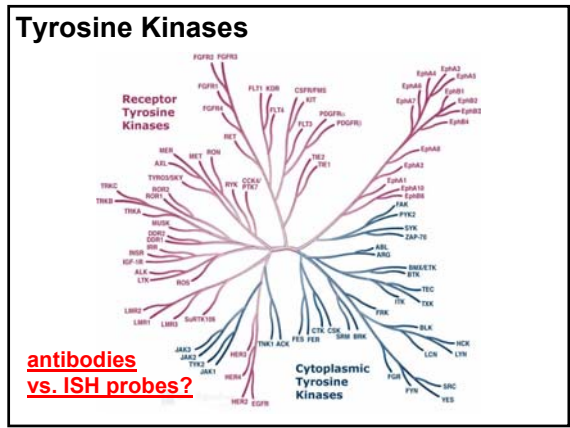
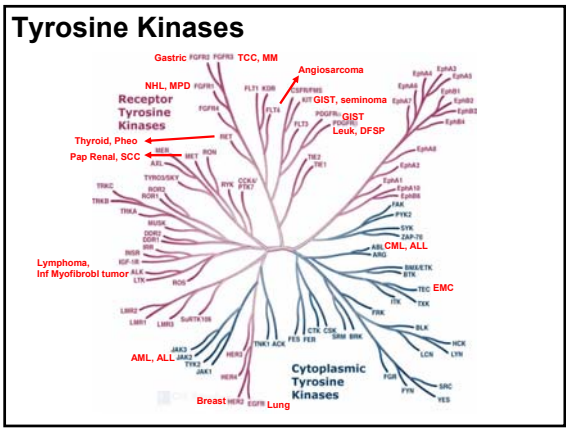
West et al, PLOS Biology, 2005, 3(6): e187.



2nd study: discovery of new fusion partners in translocation in TGCT/PVNS

TKR expression in sarcomas

- In some sarcomas, high expression of a TKR is associated with a mutation in the gene itself (KIT/PDGFRa in GIST) or a translocation in its ligand (PDGFb in DFSP).
- These TKR can function as therapeutic targets.
- Hypothesis: a screening for high TKR expression by ISH on a 420-case sarcoma TMA can identify novel therapeutic targets for other sarcomas.

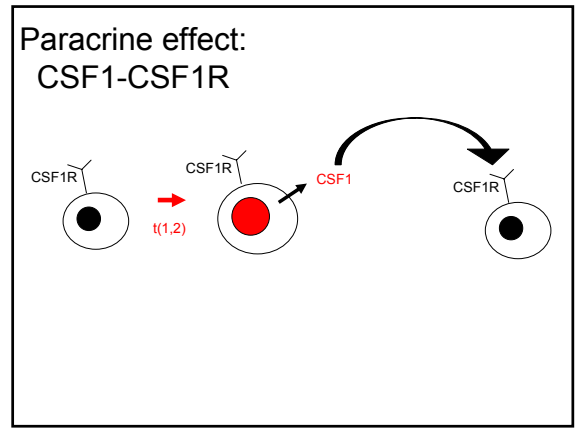
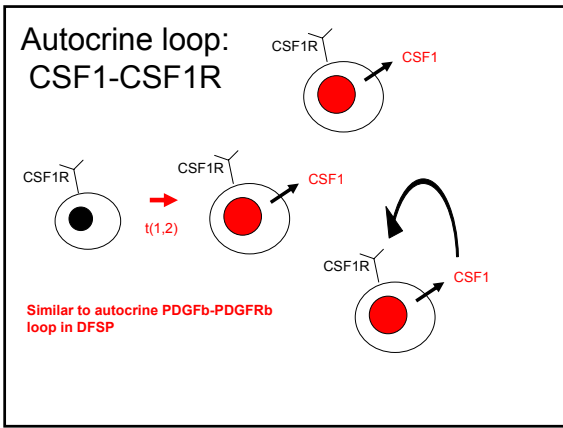
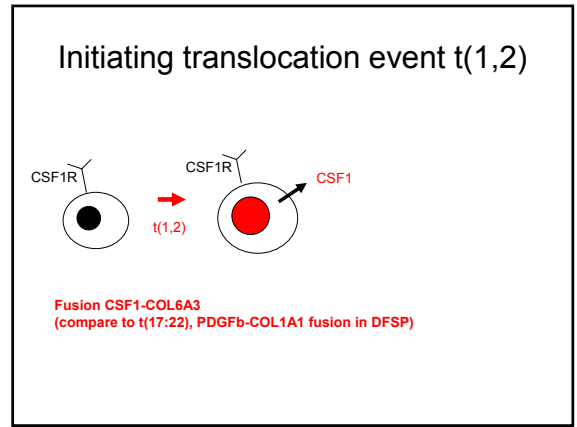
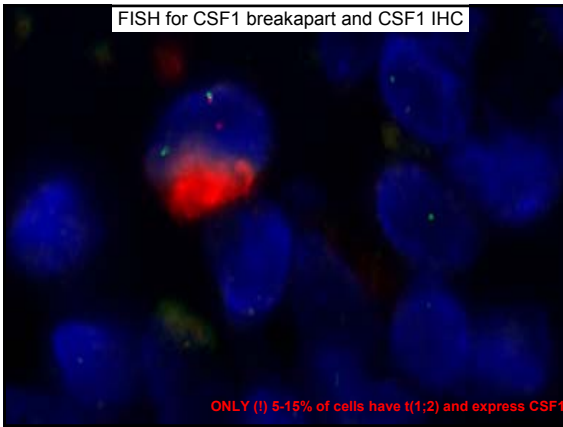
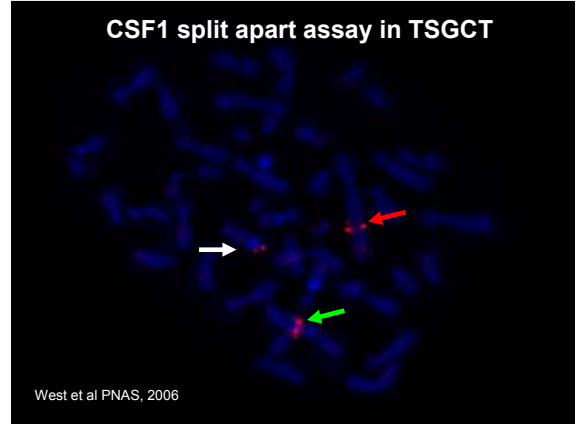


TGCT Karyotypes: Published Data

Nilsson et al, Virchow Arch, 2002

Case no.	Age (yr)	Sex	Localization	Report
1	43	Female	Brain	46,XX,t(1;2)(p13;p11),der(12)t(1;2)(p13;p11)
2	43	Female	Brain	46,XX,t(1;2)(p13;p11),der(12)t(1;2)(p13;p11)
3	43	Female	Brain	46,XX,t(1;2)(p13;p11),der(12)t(1;2)(p13;p11)
4	43	Female	Brain	46,XX,t(1;2)(p13;p11),der(12)t(1;2)(p13;p11)
5	43	Female	Brain	46,XX,t(1;2)(p13;p11),der(12)t(1;2)(p13;p11)
6	43	Female	Brain	46,XX,t(1;2)(p13;p11),der(12)t(1;2)(p13;p11)
7	43	Female	Brain	46,XX,t(1;2)(p13;p11),der(12)t(1;2)(p13;p11)
8	43	Female	Brain	46,XX,t(1;2)(p13;p11),der(12)t(1;2)(p13;p11)
9	43	Female	Brain	46,XX,t(1;2)(p13;p11),der(12)t(1;2)(p13;p11)
10	43	Female	Brain	46,XX,t(1;2)(p13;p11),der(12)t(1;2)(p13;p11)
11	43	Female	Brain	46,XX,t(1;2)(p13;p11),der(12)t(1;2)(p13;p11)
12	43	Female	Brain	46,XX,t(1;2)(p13;p11),der(12)t(1;2)(p13;p11)
13	43	Female	Brain	46,XX,t(1;2)(p13;p11),der(12)t(1;2)(p13;p11)
14	43	Female	Brain	46,XX,t(1;2)(p13;p11),der(12)t(1;2)(p13;p11)
15	43	Female	Brain	46,XX,t(1;2)(p13;p11),der(12)t(1;2)(p13;p11)
16	43	Female	Brain	46,XX,t(1;2)(p13;p11),der(12)t(1;2)(p13;p11)
17	43	Female	Brain	46,XX,t(1;2)(p13;p11),der(12)t(1;2)(p13;p11)
18	43	Female	Brain	46,XX,t(1;2)(p13;p11),der(12)t(1;2)(p13;p11)
19	43	Female	Brain	46,XX,t(1;2)(p13;p11),der(12)t(1;2)(p13;p11)
20	43	Female	Brain	46,XX,t(1;2)(p13;p11),der(12)t(1;2)(p13;p11)
21	43	Female	Brain	46,XX,t(1;2)(p13;p11),der(12)t(1;2)(p13;p11)
22	43	Female	Brain	46,XX,t(1;2)(p13;p11),der(12)t(1;2)(p13;p11)
23	43	Female	Brain	46,XX,t(1;2)(p13;p11),der(12)t(1;2)(p13;p11)
24	43	Female	Brain	46,XX,t(1;2)(p13;p11),der(12)t(1;2)(p13;p11)
25	43	Female	Brain	46,XX,t(1;2)(p13;p11),der(12)t(1;2)(p13;p11)
26	43	Female	Brain	46,XX,t(1;2)(p13;p11),der(12)t(1;2)(p13;p11)

Many TGCT (and PVNS) have t(1;2), involving 1p13, 2q35. Fusion partners unknown.



Robert West
Kelli Montgomery
Shirley Zhu
Subbaya Subramanian
Cheng-Han Lee
Inigo Espinosa

Brian Rubin
Marc van de Vijver
Dimitry Nuyten

Chris Corless
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Translating Mass Spectrometry-Based Proteomics of Malignant Lymphoma into Clinical Application

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- Proteomics is a multi-faceted approach to study the protein complement of the human genome
- Global proteomic consequences of oncogene/tumor suppressor gene expression, gene silencing using siRNAs or small molecule inhibitors can be analyzed by mass spectrometry
- Mass spectrometry-based proteomic approaches represent valuable tools to elucidate multiple signaling pathways that are deregulated in malignant lymphoma and thus play a critical role in identification of diagnostic markers and therapeutic targets

Introduction

The field of proteomics provides a powerful avenue to carry out functional studies of protein-protein interactions and characterization of signal transduction pathways. This is in large part due to advances in protein sample preparation, analytical sensitivity of mass spectrometers and improvements in instrument software and protein databases.

The recent development of multidimensional liquid chromatographic methods combined with tandem mass spectrometry (LC-LC-MS/MS) has permitted sensitive detection of thousands of low abundance proteins, membrane proteins and proteins with extreme isoelectric points (pI) from highly complex protein mixtures. The ability to perform global relative quantitative proteomics has been significantly enhanced by the advent of numerous isotope labeling approaches that are efficient in simplifying the proteome, and in combination with LC-MS/MS permit detection and quantification of proteins and peptides from very complex samples.

Furthermore, enrichment of phosphopeptides enables the discovery of novel sites of protein phosphorylation which play key roles in cell signaling and lymphomagenesis. New approaches for mining the serum and plasma proteome have been developed to enable the identification of low abundance proteins and peptides that may represent biomarkers of lymphoma.

Our laboratory has utilized mass spectrometry-based proteomic approaches to study deregulated signaling pathways in human malignant lymphoma with the aim of

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three proteins. The effect of rapamycin on the viability of cell lines derived from t(2;5)-positive ALCLs was determined by MTT assay and cell cycle analysis. Furthermore, rapamycin potently decreased the viability of SUDHL-1 cells (30% reduction by 10nM at 48 hours) and resulted in G₁ cell cycle arrest without induction of caspase-3 activity. Western blot analysis demonstrated a reduction in the level of phospho-p70S6kinase as well as 4EBP-1 levels. These results demonstrate overexpression of many proteins in the FRAP/mTOR pathway in NPM/ALK-positive ALCLs. Our data indicate that the majority of pediatric ALCLs express proteins in the FRAP/mTOR pathway and are constitutively activated. Furthermore, our *in vitro* data support the use of rapamycin as a therapeutic agent in ALK-positive ALCLs.

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identifying potential biomarkers which may be useful for diagnosis as well as novel therapeutic targets.

Hematological malignancies are characterized by non-random genetic alterations

For example, the anaplastic large-cell lymphoma (ALCL) is a distinct subtype of peripheral T-cell lymphomas harboring chromosomal translocations involving the ALK tyrosine kinase. The t(2;5)(p23;q35) chromosomal aberration resulting in overexpression of a chimeric oncogene, nucleophosmin-anaplastic lymphoma kinase (NPM-ALK), is the most common translocation found in these tumors. The resulting NPM-ALK fusion protein has been well documented as a constitutively active tyrosine kinase and the causative oncogene in t(2;5) positive ALCLs. The NPM-ALK protein plays a key role in ALCL lymphomagenesis and has been shown to cause lymphoid malignancy *in vitro* and *in vivo*.

Identification of NPM-ALK interacting proteins by tandem mass spectrometry

Proteins that interact with ALK tyrosine kinase play important roles in mediating downstream cellular signals, and are potential targets for novel therapies. Using a functional proteomic approach, we determined the identity of proteins that interact with the ALK tyrosine kinase by co-immunoprecipitation with anti-ALK antibody followed by electrospray ionization (ESI) and tandem mass spectrometry (MS/MS). A total of 46 proteins were identified as unique to the ALK immunocomplex using monoclonal and polyclonal antibodies while 11 proteins were identified in the NPM immunocomplex. Previously reported proteins in the ALK signal pathway were identified including PI3-K, Jak2, Jak3, Stat3, Grb2, IRS and PLCγ1. More importantly, many proteins previously not recognized to be associated with NPM-ALK, but with potential NPM-ALK interacting protein domains were identified. These include adaptor molecules (SOCS, Rho-GTPase activating protein, RAB35), kinases (MEK kinase 1 and 4, PKC, MLCK, cyclin G-associated kinase, EphA1, JNK kinase, MAP kinase 1), phosphatases (mepirin, PIPK, protein phosphatase 2 subunit) and heat shock proteins (Hsp60 precursor). Proteins identified by MS were confirmed by western blotting and reciprocal immunoprecipitation. This study demonstrates the utility of antibody immunoprecipitation and peptide identification by nanoflow ESI-LC/MS/MS for the high-throughput identification of proteins within the ALK signaling complex and potential definition of its signaling pathways.

Quantitative proteomic analysis of differentially expressed proteins induced by NPM-ALK overexpression

The global molecular and cellular consequences of NPM-ALK overexpression are largely unknown. In addition, the identity and function of only a limited number of downstream molecules important for its oncogenic activity are currently understood. We have used a functional quantitative proteomic approach to determine the global effects of NPM-ALK expression on cell function. Jurkat cells were transfected with a plasmid containing the NPM-ALK chimeric gene. The NPM-ALK overexpressing Jurkat cells were compared to those expressing the vector only. Quantitative analyses of differentially expressed

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proteins were determined by isotope-coded affinity tagging (ICATTM) followed by liquid chromatography (LC) and tandem mass spectrometry (MS/MS). Equivalent quantities of total cell lysates obtained from the NPM-ALK transfected cells and the vector control cells were ICATTM labeled, and subjected to avidin affinity chromatography. Offline fractions were collected, digested with trypsin, and analyzed by automated reverse phase nanospray LC-MS/MS. Some 124 proteins showed a 1.5 fold or greater change in the NPM-ALK positive cells as compared to the vector control cells. Of these, 79 proteins were upregulated by greater than 1.5-fold while 45 proteins were downregulated by greater than 1.5-fold. Differential expression of selected proteins was validated by western blot analyses. Analysis of functional groups of proteins demonstrated upregulation of protein kinases, cytoskeletal proteins and proteins associated with proliferation and translation. In addition, upregulation of proteins previously reported to be important mediators of the ALK signaling pathway including, PLCγ1, Ki-67, GRB2, Jak2, and PI3-K were observed. Our studies reveal the global proteomic consequences of NPM-ALK overexpression as a singular molecular abnormality and provides novel insight into the signal transduction pathways influenced by cellular transformation by NPM-ALK. Interestingly, several proteins associated with cytoskeletal organization were selectively expressed in ALK-positive ALCLs and not in ALK-negative anaplastic large cell lymphoma or in classical Hodgkin lymphoma. These proteins represent potential markers that may help in distinguishing between entities within CD30+ lymphoproliferative disorders.

Constitutive activation of FRAP/mTOR pathway in pediatric anaplastic large cell lymphomas: Potential role as a therapeutic target

Our quantitative proteomic studies identified many proteins which were downstream targets of the FRAP/mTOR pathway including ribosomal S6 kinase (1.6-fold), translational initiation factor eIF (4.8-fold), ribosomal protein L11 (4.8-fold), eukaryotic translation initiation factor 3 (3.2-fold), translation initiation factor IF-2 homolog (4.3-fold) and translation initiation factor eIF-2α kinase (3.4-fold). The FRAP/mTOR pathway plays a key role in the regulation of cell growth and proliferation and positively regulates translation and ribosome biogenesis and is selectively inhibited by rapamycin.

To determine the feasibility of targeting the FRAP/mTOR pathway by rapamycin for treatment of pediatric ALCLs, we determined the prevalence of expression of key proteins in the FRAP/mTOR pathway in pediatric ALCLs and correlated its expression with that of the ALK protein. In addition we determined the *in vitro* effect of rapamycin on the viability of cell lines derived from t(2;5)-positive ALCLs.

Formalin-fixed paraffin-embedded tissues of ALK-positive ALCLs were used to determine the expression of phospho-mTOR, phospho-70S6kinase and phospho-S6 ribosomal protein using immunohistochemistry. Immunohistochemical studies demonstrated diffuse strong nuclear expression of phospho-mTOR in 17/18 cases, and nuclear and cytoplasmic phospho-70S6kinase expression in 15/18 cases. In addition, cytoplasmic expression of phospho-S6 ribosomal protein was observed in 18/18 (100%) of cases. Importantly, the reactive lymphocytes demonstrated negligible expression of all

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4:15 **Molecular Profiles of Follicular Cell Thyroid Tumors (Approx. 40 minutes with 5 minutes for questions)**
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Abstract

Well-differentiated thyroid carcinoma is a common type of thyroid cancer that is increasing in incidence. The cytologic and pathologic diagnosis of thyroid follicular tumors is often challenging, resulting in thousands of unnecessary surgeries for benign disease and disagreement amongst pathologists. More objective diagnostic tools would help reduce these difficulties. Further, in this age of targeted therapy, improved understanding of follicular cell thyroid cancer and its progression to aggressive forms of thyroid cancer would be beneficial for the development of appropriate novel therapies. In this presentation, some recent translational work using gene expression profiling approaches is presented.

Introduction

Follicular cell thyroid carcinoma is broadly divided into papillary (PTC) and follicular types (FTC). This distinction is based primarily on their morphologic overall features, i.e. tumor architecture and characteristic nuclear features (e.g., optical clearing). The classification is clouded by the recognition of variants such as the follicular variant of papillary carcinoma (FVPTC) and the subjectivity inherent in morphologic assessment. Consequently, there persists a high degree of diagnostic intra- and inter-observer variability¹. One of the goals of genomic molecular pathology approaches is to reduce the diagnostic subjectivity of follicular cell tumors². Over the past few years, several laboratories, including ours, have begun to use genomic approaches to address several issues in thyroid follicular cell pathology, such as understanding pathogenesis and improved diagnosis and prognosis. This presentation will highlight some work in this field.

Gene expression profiling recapitulates the overall classification of thyroid tumors

Using commercially-available oligonucleotide DNA microarrays (Affymetrix U133A), our laboratory has generated a gene expression dataset that corresponds to the common types of benign and malignant thyroid tumors. Various clustering methods have been applied to this dataset derived from 99 thyroid samples, resulting in a molecular classification that recapitulates the overall morphologic classification of thyroid tumors (Figures 1 and 2). This observation provides strong and compelling validation of the microarray data. The result from the first method, principal component analysis (PCA), is shown in Figure 1.



Figure 1. Principal components analysis (PCA) of 99 thyroid samples (96 tumors and 4 normal thyroids). The PCA reveals separation of the papillary carcinomas (brown), the anaplastic carcinomas (red), and defines a follicular patterned cohort that includes normal thyroid (dark blue), follicular adenoma (black), follicular carcinoma (green). Further, the oncocytic tumors (purple and yellow) also define a separate group. Two medullary carcinomas are distinctly different (light blue).

The result from the second clustering method, hierarchical clustering (HC), is shown in Figure 2. In addition to morphologic assessment, the tumors were genotyped for their common mutations (e.g. BRAF, RET/PTC, RAS, PAX8-PPARgamma translocation). These results from the PCA and HC along with morphologic assessment reveals some interesting observations and confirms some previously known relationships, as outlined below:

1. There is a large degree of heterogeneity within the PTC cohort. This is consistent with the numerous morphologic variants associated with PTC and several different activating mutations (BRAF, RET/PTC, and RAS) (Figure 1).
2. Follicular patterned lesions (FA, FC and normal thyroid) share similar global patterns of gene expression.
3. Oncocytic tumors, benign and malignant, share global gene expression profiles.
4. Anaplastic carcinoma has a distinctly different gene expression pattern.
5. Medullary carcinoma has a distinctly different gene expression pattern.
6. Hierarchical clustering divides the cohort generally into tumors with papillary architecture and follicular architecture (Figure 2.)
7. PTC with BRAF activating mutations form several subclusters with the papillary architecture cluster (Figure 2).
8. PTC with RET/PTC mutations form 2 subclusters within the papillary architecture cluster (Figure 2).
9. PTC, follicular variants, form 2 subclusters within the follicular patterned tumors and have a predominance of RAS mutations.
10. FTC with the PAX8-PPARgamma translocation form a distinct subcluster within the follicular patterned lesions (Figure 2).
11. Normal thyroid shares a similar global gene expression profile within the follicular patterned samples (Figure 2).
12. Separation of many of the follicular patterned tumors is not straightforward (Figure 2).

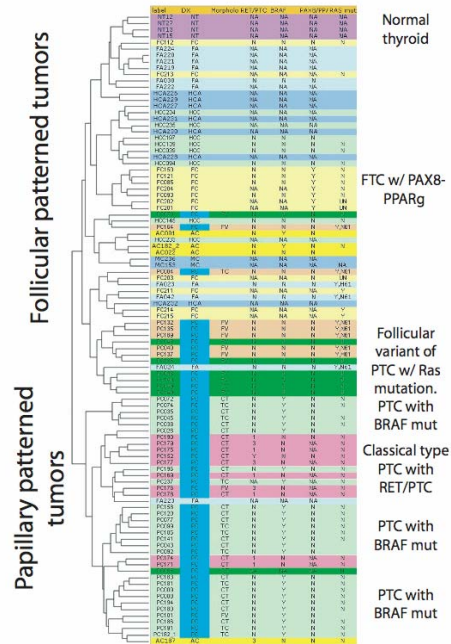


Figure 2. Hierarchical clustering of 99 thyroid samples divides the tumors into 2 broad groups, i.e. tumor with papillary architecture and tumors with follicular architecture.

Activating mutations of the MAPK pathway are the primary determinant of gene expression variation within PTC

Examination of the PTC cohort by PCA revealed a molecular classification of PTC that was a reflection of both tumor morphology and underlying activation mutation³, as summarized in Figure 3.

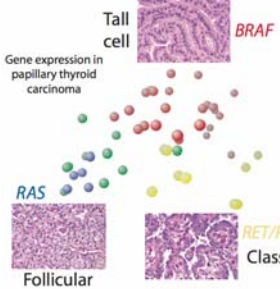


Figure 3. PCA of 51 PTC samples showing the relationships between tumor morphology and gene expression and mutation and gene expression. PTC with BRAF (red) tend to form a distinct cluster and have a tall cell morphology. PTC with RET/PTC rearrangements (yellow) tend to form a distinct cluster and have a classic papillary morphology. PTC with RAS mutations tend to form a distinct cluster (blue) and have a follicular morphology.

This striking observation means that these mutations are the primary determinants of gene expression variation in these tumors. The broader implication is that PTC is a relatively simpler carcinoma compared to many other common epithelial tumors, e.g. lung adenocarcinoma, in which it is much harder to define a relationship between mutation and gene expression. The implication of this observation is that PTC should be amenable to treatment with targeted therapies that block these activating mutations. Clinical trials are underway with specific BRAF inhibitors and it will be interesting to see their results. Animal studies suggest the BRAF inhibition will be effective⁴.

Follicular carcinoma with the PAX8-PPARgamma translocation have distinct gene expression profiles

Included in our dataset are 7 FTCs with the PAX8-PPARgamma translocation. This molecular alteration was first described in 2000 in a subset of FTCs⁵ and the observation has since been confirmed and extended by a number of studies⁶⁻⁹. Several groups have examined gene expression in these tumors¹⁰⁻¹², with the 2 most recent studies in general agreement that these tumors display a characteristic gene expression profile. The latter 2 studies revealed that FTC with PAX8-PPARgamma translocation have a gene expression profile that is a function of the activity of the fusion protein, called PFPF. These studies have implications for the role of this fusion protein in the pathogenesis of this type of FTC and by extension the treatment of this tumor. These aspects were recently reviewed¹³.

Potential of molecular profiling for thyroid tumor diagnosis and prognosis

Many studies have examined the potential of molecular profiling for thyroid cancer. While none of the studies have resulted in a clinically available and validated diagnostic assay, the results are generally encouraging. For instance, a recent study by Lubitz et al. showed that gene expression profiling could be accurately employed at the cytology level¹⁴. Using a small cohort of 22 FNA specimens, they showed that examining 25 differentially expressed genes could correctly classify these lesions using hierarchical clustering. Although this study needs to be extended to a larger cohort and hierarchical clustering is not recommended for diagnostic assays¹⁵, it illustrates the potential of genomic approaches especially if they can be applied at the pre-operative level.

Other studies have addressed the issue of genomic prediction of prognosis, a difficult task given the overall excellent prognosis of thyroid carcinoma. One such study used a genomic approach to delineate the MUC1 gene as an independent prognostic factor in PTC in a multivariate analysis¹⁶.

MicroRNA profiling

MicroRNA profiling represents a complementary approach to messenger RNA profiling. MicroRNAs are small RNA molecules that are thought to function as negative regulators of gene expression¹⁷. They possess significant diagnostic potential^{18,19} and have begun to be examined in thyroid cancer^{20,21}. In addition to a role in PTC, our study of FTC with PAX8-PPARgamma translocation showed altered expression microRNA target genes, suggesting that microRNAs may play a role across the full spectrum of thyroid tumors. Much work still needs to be done in this exciting and promising area.

Conclusion

The potential of using molecular profiling for thyroid neoplasia is significant. Such approaches have and should result in:

1. Improved understanding of thyroid tumor pathogenesis.
2. Improved understanding of thyroid cancer progression to aggressive forms.
3. Refinement of thyroid tumor classification.
4. Identification of novel diagnostic markers.
5. Identification of novel prognostic markers.

Collectively, these advances should result in improved management and care of patient with thyroid nodules and cancer.

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