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TITLE: Genomic and Expression Profiling of Benign & Malignant Nerve Sheath Tumors
in Neurofibromatosis Patients

PRINCIPAL INVESTIGATOR: Matt van de Rijn, M.D., Ph.D.
Torsten Nielsen, M.D., Ph.D.
Brian Rubin, M.D., Ph.D.

CONTRACTING ORGANIZATION: Leland Stanford Junior University
Stanford, California 94305-4125

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14. ABSTRACT The goal of the study is to identify genes and pathways that are associated with the progression of neurofibroma to MPNST, and to identify potential therapeutic targets for MPNSTs. In the past year, to the existing 80 gene array data, we added an additional 23 tumor samples making to a total of 103 gene arrays. The analyses included 38 cases diagnosed as MPNSTs and 24 cases of neurofibromas. Our initial hierarchical clustering showed high degree of variability of MPNST cases. A centralized review of histology was performed; of the 38 cases with the original diagnosis as MPNST, 14 cases were reclassified. In addition all MPNST and SS cases underwent RT-PCR for t(X;18). The misdiagnosed tumor cases were removed from the study, and a subsequent analysis and hierarchical clustering showed a much more interpretable result. The clustering of the nerve sheath tumors revealed a distinct expression signature for majority of benign and malignant tumor types. A subset of MPNSTs clustered along with SS. Using a large tissue microarray (TMA) with about 200 nerve sheath tumors we have identified a novel diagnostic marker TLE1 to distinguish SS from other sarcomas. The signaling pathways TGFB, JAK- STAT, MET were identified as being potentially involved in the malignant transformation. Array comparative genomic hybridization was performed on 28 tumor cases and copy number changes were assessed for each case.					
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Table of Contents

Cover.....	1
SF 298.....	2
Introduction.....	4
Body.....	5
Key Research Accomplishments.....	11
Reportable Outcomes.....	11
Conclusions.....	12
References.....	14
Appendices.....	34

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Title: Genomic and Expression Profiling of Benign and Malignant Nerve Sheath Tumors in Neurofibromatosis Patients.

INTRODUCTION

Nerve sheath tumors generally develop in the setting of neurofibromatosis (NF1), affecting 1 in 3000 individuals worldwide¹. The most common tumor in NF1-patients is the benign 'neurofibroma'. However, about 8-13% of NF1 patients have a risk of developing malignant peripheral nerve sheath tumors (MPNSTs)². MPNSTs are aggressive neoplasms that may also arise sporadically.^{3,4} Malignant transformation is a life threatening complication. The development of neurofibromas and MPNSTs involves mutations of multiple tumor suppressor genes such as NF1⁵. However, it is widely believed that mutations in tumor suppressors alone are not enough to induce peripheral nerve sheath tumor formation⁶⁻⁸.

The objective of the study is to identify gene(s) that will serve as a molecular marker for patients in which the benign neurofibroma is progressing towards MPNST and to identify novel therapeutic targets for MPNSTs. We have carried out the expression profiles for over 103 nerve sheath tumors. Our initial hierarchical clustering showed high degree of variability of MPNST cases. The histological diagnosis of MPNST is difficult and often arbitrary. Our samples were obtained from a large number of collaborators and we decided to have a centralized review of histology to be performed by Dr Christopher Fletcher at Brigham & Women's Hospital, Boston. Of the 38 cases submitted as MPNST, 14 cases were reclassified. In addition all MPNST and synovial sarcoma (SS) cases underwent RT-PCR for t(X;18). After these analyses, 24 cases of classical MPNSTs and 13 cases of synovial sarcoma remained. Subsequent analysis and hierarchical clustering showed a much more interpretable result. The nerve sheath tumors revealed distinct expression signature for benign and malignant tumor types. A subset of MPNSTs clustered along with SS. Synovial sarcomas pose a major challenge in the correct diagnosis of MPNSTs. We created a large tissue microarray (TMA) with about 200 nerve sheath tumors and identified a novel diagnostic marker (TLE1) to distinguish SS from other sarcomas including MPNSTs (Terry et al., in press).

BODY

Specific aim 1: Genome wide search for genes in nerve sheath tumor

A. Gene expression profiling

A1. Collection and gene array analyses of additional cases

In the past year, to the existing gene array data on 80 nerve sheath tumor samples, we performed gene array profiling on additional 23 tumor samples. A total of 103 gene arrays were analyzed using various statistical programs. The unsupervised hierarchical clustering showed high degree of variability of MPNST cases. Initial gene array analyses of cases submitted as MPNSTs and SS yielded incomprehensible results. Diagnosis of MPNSTs poses a major challenge to pathologists; especially the histological/morphological distinction of MPNSTs from SS is a difficult task. We decided to do a centralized review of all the 54 MPNST and SS cases. Towards this, we collected the paraffin tissue blocks of all most all the MPNSTs and SS tumor samples that were included in the study.

A2. Centralized review of MPNST and SS tumor samples

A centralized review of histology for all MPNSTs and SS cases was performed by Dr Christopher Fletcher at Brigham & Women's Hospital, Boston. HE slide along with various immunostains performed on 6 unstained slides were used to review the MPNST and SS cases. We also performed RT-PCR using the SSX-SYT primers on all the MPNST and SS cases for detecting the t(X;18). The misdiagnosed tumor cases were removed from the study, and a subsequent analysis and hierarchical clustering showed a much more interpretable result.

A3. Gene expression profiling of nerve sheath tumors

In total, 87 tumor samples were used for gene array analysis, including 24 MPNSTs, 28 neurofibromas, 22 schwannomas and 13 synovial sarcomas. The microarrays used in the study contain a total of about 42000 cDNAs representing about 28 000 genes or ESTs. Only cDNA spots with a ratio of signal over background of at least 2 in either the Cy3 or Cy5 channel were included; only genes with 80% good data were included. Genes were

further selected when expression levels differed by at least four-fold in at least three arrays. Using these criteria, 5540 genes passed the filtering criteria and were used for further analysis. Unsupervised hierarchical clustering analysis⁹ and significance analysis of microarrays [SAM]¹⁰ were then performed as described previously¹¹. Unsupervised analysis using average-linkage clustering, grouped the 87 tumor cases into two main groups. Most of the malignant tumors i.e MPNSTs and SS grouped in branch 'A' leaving the majority of the benign neurofibromas and schwannomas in a distinct branch 'B' (Figure 1).

Further analysis of tumor cases in branch 'A' revealed that MPNSTs and SS formed discrete groups. While the separation was not absolute most SS clustered together. A small subset of MPNSTs clustered along with SS. Interestingly the MPNSTs that clustered along with SS were all derived from male patients. The X chromosome inactivation gene '*XIST*' showed a decreased expression in those MPNST cases that clustered with SS. In contrast, *XIST* was highly expressed in all most all female patient samples included in this project. Synovial sarcoma is characterized with a translocation involving chromosomes X and 18 this may have some influence on clustering. However, at this point we have no clear explanation for this.

A4. Supervised analysis using significance analysis of microarrays

We subsequently analyzed the expression data by SAM, to identify and rank order the genes that differentiate various nerve sheath tumors based on their gene expression profiles. Using data from all 87 arrays, four SAM analyses were performed. First, we compared all the MPNSTs to the other tumors. Second, we analyzed genes that separated neurofibromas from the other cases. Third, we investigated the genes specifically expressed in schwannomas. Finally, the genes that distinguished SS from the other tumors were identified. The partial lists of highest ranking genes identified in these four separate SAM analyses are shown in Tables 1a, 1b, 1c and 1d respectively.

A.5. Malignant transformation in MPNSTs

A5.1. Consequences of malignant transformation in MPNSTs

The major trend in transformation from neurofibroma towards MPNST is the loss of expression of a large number of genes, rather than widespread *de novo* increase in gene expression (Figure 1). *NF1* is one of the genes for which loss of expression was noticed in most of the MPNSTs. Down regulation of large number of genes upon transformation may suggest a role for epigenetic mechanisms such as DNA hypermethylation. Further, several transcription factors including *ETS1*, *NFATC2* and *JUN* were down regulated in most MPNSTs. Examples of genes and pathways affected by transformation are shown below.

A5.2. Loss of p53 function

Inactivation of p53 serves as characteristic feature in the process of malignant transformation. As a consequence of p53 inactivation, several genes that are regulated by p53 mediated signaling are up regulated. We analyzed the expression patterns of ‘inactivated p53 associated proliferation signature’ genes¹² in the neurofibromas and MPNSTs. The gene expression profile analyses in nerve sheath tumors revealed that several ‘p53 inactivation signature genes’ such as *TOP2A*, *TTK*, *CDC2*, *CDC25A*, *HMMR*, *PTTG3* and *UBE2C* were up regulated in MPNSTs (Figure 2). Though most MPNSTs included in the analysis showed the signature for p53 inactivation, four MPNST tumor cases STT4734, STT4737, STT4541 and STT3920 did not show the signature for p53 loss (Figure 2). This observation suggests the presence of additional mechanisms that might lead to transformation.

A5.3. Proliferation signature

The proliferation signature includes genes responsible for the cell cycle transcriptional regulation and replication initiation complex. The proliferation signature is associated with a core set of genes such as *MCM2*, *MCM4*, *MCM6*, *BUB1*, *PLK1*, *CCNB2*, *TOP2A*, *FOXM1* and *MK167*¹³. Evaluating the expression profile for the core set of proliferation genes in MPNSTs and other tumors revealed that the proliferation signature was

predominantly associated with MPNSTs and SS and not with the benign tumors, neurofibroma and schwannoma (Figure 3). The proteins encoded by MCM gene family are involved in the initiation of eukaryotic genome replication and their increased expression is correlated with tumor cell proliferation¹⁴. MPNSTs showed increased expression for genes *MCM2*, *MCM4* and *MCM6* (figure). The MCM proteins are regulated by, protein kinases CDC2 and CDC7. Expression of *CDC2* and *CDC7* are highly correlated with the expression of MCM genes (Figure 3).

A5.4. Loss of cell adhesion and metastasis signature

Tumor invasion is generally associated with loss of expression of genes such as E-cadherin and over expression of the zinc-finger transcriptional repressor gene ‘snail’¹⁵⁻¹⁷. In our analysis we notice the down regulation of E-cadherin (*CDH1*) in both neurofibroma and MPNSTs. Additional cell adhesion genes such as CD44, and integrins that show differential expression among nerve sheath tumors are shown in Figure 3. *TWIST1*, *UBE2C* and *HMMR* are highly expressed in MPNSTs, these genes are known to play a role in malignant progression¹⁸⁻²⁰.

A6. Gene networks in MPNSTs

We have identified several gene networks (genes and its interacting partners that are functionally associated) that are differentially expressed in MPNSTs. As an example, here we discuss one of the gene networks.

Msh homeobox homologs *MSX1* and *MSX2* are highly expressed in majority of MPNSTs. These transcriptional repressors have a role in the differentiation and survival of cells. Yoshizawa et al²¹ established that *MSX2* co-localizes with *Runx2* and *Osf2* (*POSTN*) and suppresses its activity cooperatively, acting with another corepressor, *TLE1*. Our study reveals that SS expresses abundant *TLE1* transcript compared to MPNSTs. Consistent to the earlier findings, in the absence of the co-repressor *TLE1*, expression of *POSTN* and *RUNX2* are predominantly seen in MPNSTs but not in SS. We have shown that *TLE1* can be used as a diagnostic marker which can distinguish SS from MPNSTs (see below).

A7. Specific signaling pathways affected during malignant transformation in MPNSTs

To find the specific signaling pathway(s) that could be affected during malignant transformation, we carried out gene set enrichment analysis [GSEA] ²². The program identifies genesets that are enriched in a rank ordered gene list. By comparing the expression profile of neurofibroma with MPNST, we identified several signaling pathway that could be potentially associated with the malignant transformation. Gene associated with signaling pathways such as TGFB, MET, NFKB and JAK-STAT were down regulated in MPNSTs. A list of signaling pathways and the enriched genes found that show differential expression is shown in table 2 and tables 3a-3e.

Aim 1. B: Array Comparative Genomic hybridization (aCGH)

B1. aCGH analysis of nerve sheath tumors

Using the same cDNA array platform that we used for the expression analysis, we evaluated the copy number changes of over 30 nerve sheath tumors. After the final histological diagnosis of the MPNSTs and SS, 28 arrays were selected for further analysis which includes 11 MPNSTs, 6 neurofibromas, 5 schwannomas and 6 synovial sarcomas. Tumor DNA from frozen tissue and reference DNA (normal gender-matched human leukocytes) were extracted and used in the aCHG analysis. Of the over 43,000 cDNA sequences represented on the microarrays used for this study, the chromosomal localization is known for about 21500 genes/transcripts. For CGH data the copy number for each locus was based on a moving average of the five nearest cDNA clones centered on that locus. A representative caryoscope analysis on a MPNST case which shows the gain of chromosome region 7p is shown in figure 4. A snapshot of 7p21.1 genomic locus amplification in majority of MPNSTs, which harbors *TWIST1*, is shown in Figure 5.

Specific Aim 2: Validation of candidate genes on large numbers of cases using immunohistochemistry and *in situ* hybridization on TMA.

A1. Construction of nerve sheath tumor tissue array

In the previous annual report we described the construction of a tissue microarray (TA-138) containing 68 MPNSTs, 42 neurofibromas, 22 schwannomas and 15 synovial

sarcomas. To our knowledge, this remains the largest TMA of nerve sheath tumors. In the past year we have made in situ hybridization probes for several differentially expressed genes in nerve sheath tumors such as *FGFR1*, *FGFR2*, *FGFR3*, and *CD44*. Analysis of these and other genes on TA-138 is ongoing. Using immunohistochemistry (IHC) we have analyzed the expression of EGFR in nerve sheath tumors. Consistent with the gene array findings our TMA analyses revealed that a subset of MPNSTs show strong expression of EGFR. Representative MPNST staining for EGFR is shown in Figure 6. Further we have titred many antibodies (*ZIC1*, *TTK*, *L1CAM*, *ERBB3*, *TLE3*, *PLA2R1*, *TNSF10*, *PTTG1*, *TBX3*, *FAT*, *TFP1*, and *PLA2G2A*) to allow staining on TA-138. We have managed to collect the paraffin tissue blocks for all most all the MPNST and SS cases that were used in our gene array study. From these paraffin blocks a tissue microarray will be constructed. Staining this TMA with potential biomarkers selected from our gene array studies will serve as a direct confirmation for our gene array data.

A2. TLE1 as a novel diagnostic marker for synovial sarcoma

Synovial sarcoma is a soft tissue malignancy defined by the SYT-SSX fusion oncogene. Gene expression profiling studies performed by us and other groups have consistently identified TLE1 as an excellent discriminator of synovial sarcoma from other sarcomas, including histologically similar tumors such as MPNSTs. TLE proteins (human homologues of groucho) are transcriptional corepressors that have an established role in repressing differentiation. We examined the expression of TLE proteins in SS and in a broad range of mesenchymal tumors using tissue microarrays to assess the value of anti-TLE antibodies in the immunohistochemical confirmation of synovial sarcoma. We have demonstrated that TLE expression is a consistent feature of SS. TLE shows intense and/or diffuse nuclear staining in 91/94 molecularly-confirmed synovial sarcomas. In contrast, TLE staining is detected much less frequently and at lower levels, if at all, in 40 other mesenchymal tumors. (Please see the attached manuscript in press for details)

KEY RESEARCH ACCOMPLISHMENTS

1. Gene expression profiling on a large number (87 cases) of nerve sheath tumors and the related lesion synovial sarcoma
 - Bioinformatical and statistical analyses to characterize molecular events that are potentially associated with malignant transformation.
 - Identification of genes and gene networks that are potentially associated with malignant transformation.
2. Array comparative genome hybridization.
3. Development of diagnostic marker for synovial sarcoma

REPORTABLE OUTCOMES:

Abstract

Subramanian S, West RB, Nielsen TO, Rubin BP, Downs-Kelly E, Goldblum JR, Zhu S, Montgomery K, Hogendoorn PCW, Corless CL, Oliveira AM, Fletcher CDM and van de Rijn M. Genome-wide transcriptome analysis of nerve sheath tumors (2006) 97th Annual meeting of American Association of Cancer Research AACR Washington DC. USA (Poster)

Publication

Terry J, Saito T, Subramanian S, Cindy R, Antonescu CR, Goldblum JR, Downs-Kelly E, Corless CL, Rubin BP, van de Rijn M, Ladanyi M, and Nielsen TO. TLE1 as a diagnostic immunohistochemical marker for synovial sarcoma emerging from gene expression profiling studies. 2006 *Am J Surg Path* (in press)

CONCLUSIONS

In the past year we have performed gene microarray experiments on a significant number of additional cases of MPNSTs, neurofibromas, schwannomas, and synovial sarcomas for a total of 103 specimens. Correct diagnosis of MPNSTs poses a major challenge to pathologists; especially the histological/morphological distinction of MPNSTs from SS is a difficult task. In order to use MPNST and SS tumor samples with correct diagnosis in our gene expression studies, we have spent considerable amount of time in collecting and organizing the paraffin tissue blocks for all the 54 MPNST and SS cases that were analyzed by gene arrays.

Dr Christopher Fletcher kindly agreed to review all the MPNST and SS cases. It can be appreciated that the gene expression data after the centralized review by Dr Fletcher is much cleaner and appealing as compared to the last year's clusters that we submitted in our 2005 annual reports. As a further , molecular confirmation of the diagnosis, we determined the presence or absence of the SSX-SYT fusion transcript for all most all cases of MPNSTs and SS by RT-PCR. For those cases where no RNA was available, fluorescence in situ hybridization (FISH) was performed on paraffin sections to detect the X:18 translocation. We have carried out considerable amount of data analyses, literature searches and pathway analysis to characterize the molecular events that are associated with malignant transformation.

In the past year we also performed array comparative genomic hybridization experiments for 28 cases nerve sheath tumor cases including 6 SS. We identified loss and gain of genomic regions in nerve sheath tumor types. Many of the chromosomal aberrations that we noticed have previously been reported in the literature. We noticed that in a subset of MPNSTs, amplification of the locus 7p21 occurs. This locus harbors *TWIST1*, the gene responsible for tumor metastasis. In addition to testing several antibodies on TMA for their potential to be a diagnostic marker, we have constructed several ISH probes that are currently being studied.

In an extraordinary collaboration among the three collaborating institutions on this grant (Stanford University Medical Center, University of British Columbia, University of Washington), we have been able to generate and analyze a significant amount of gene array and aCHG data. We have presented a portion of our findings in the annual meeting

of American association for cancer research and were able to submit a manuscript that describes a novel diagnostic marker for SS. Currently we are writing up the manuscript on the gene array analysis of nerve sheath tumors that will be soon be submitted for publication.

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LEGENDS FOR FIGURES

Figure 1

Unsupervised hierarchical cluster analysis of gene expression profiles of 87 nerve sheath tumors and SS along with the heat map of the 5540 genes used for generating hierarchical clustering. Each row represents the relative levels of expression for a single gene. Each column shows the expression levels for a single sample. The red or green color indicates high or low expression, respectively.

Figure 2

Expression profiles of genes that are associated with the inactivation of p53.

Figure 3

Overview of expression pattern of selected genes involved in cell cycle regulation, cell adhesion, metastasis and gene networks etc.

Figure 4

A representative caryoscope generated for MPNST by determining loss or amplification in chromosomes (CLAC) using CGH-miner program.

Figure 5

A screenshot showing the copy number increase in p-arm of chromosome 7 and the associated genes in that locus.

Figure 6

Representative cores showing strong positive and negative immunohistochemistry staining of EGFR in MPNSTs. a) Strong expression of EGFR in MPNST; b) absence of EGFR expression in MPNST.

LEGEND FOR TABLES

Table 1a

A partial list of genes with highest differential expression in MPNSTs according to SAM analysis.

Table 1b

A partial list of genes with highest differential expression in neurofibromas according to SAM analysis.

Table 1c

A partial list of genes with highest differential expression in schwannomas according to SAM analysis.

Table 1d

A partial list of genes with highest differential expression in synovial sarcomas according to SAM analysis.

Table 2

Signaling pathways identified by gene set enrichment analysis method that are affected during the malignant transformation.

Table 3a-e

Signaling pathway and the associated genes that show differential expression in our gene array analyses.

Figure 1

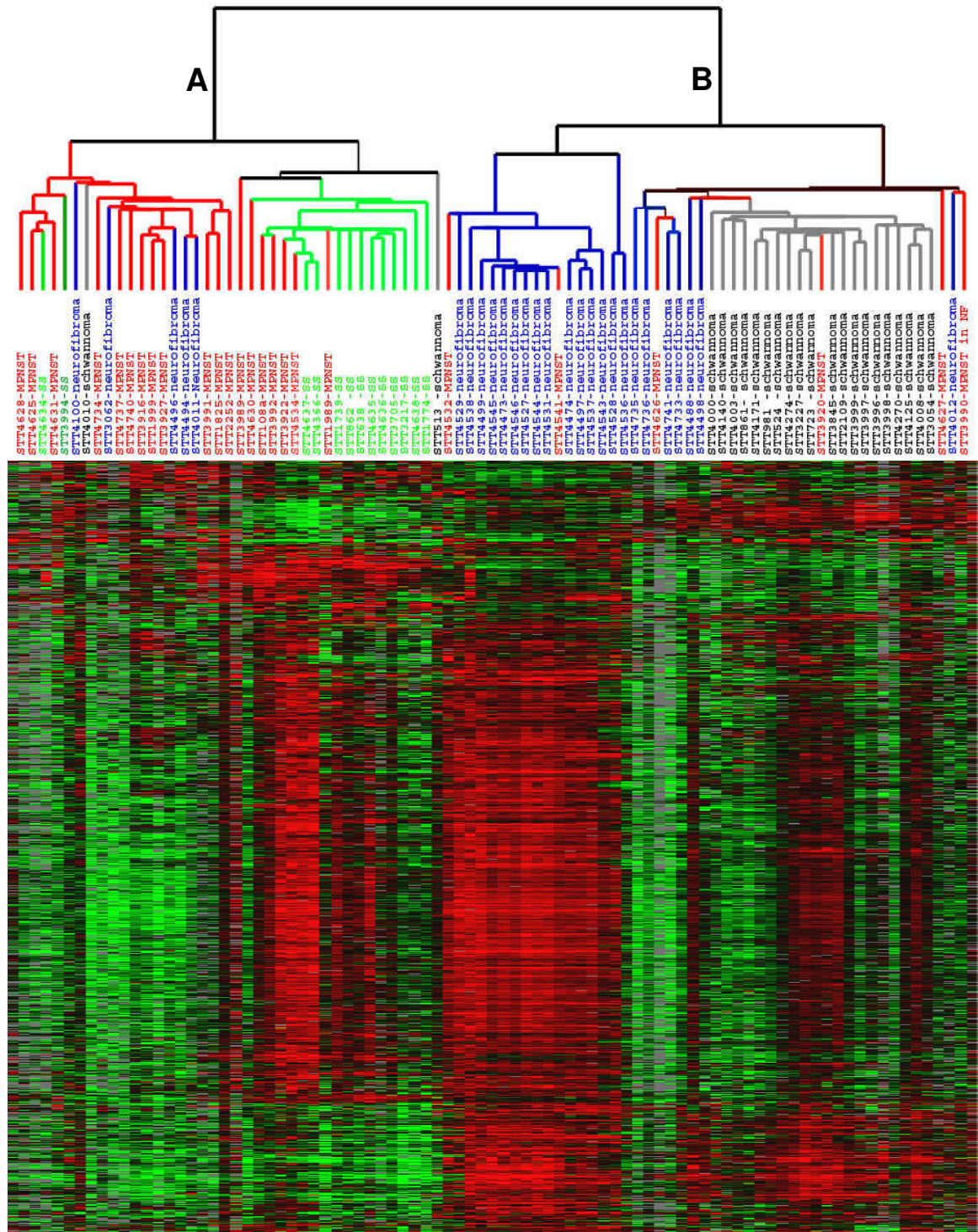


Figure 2

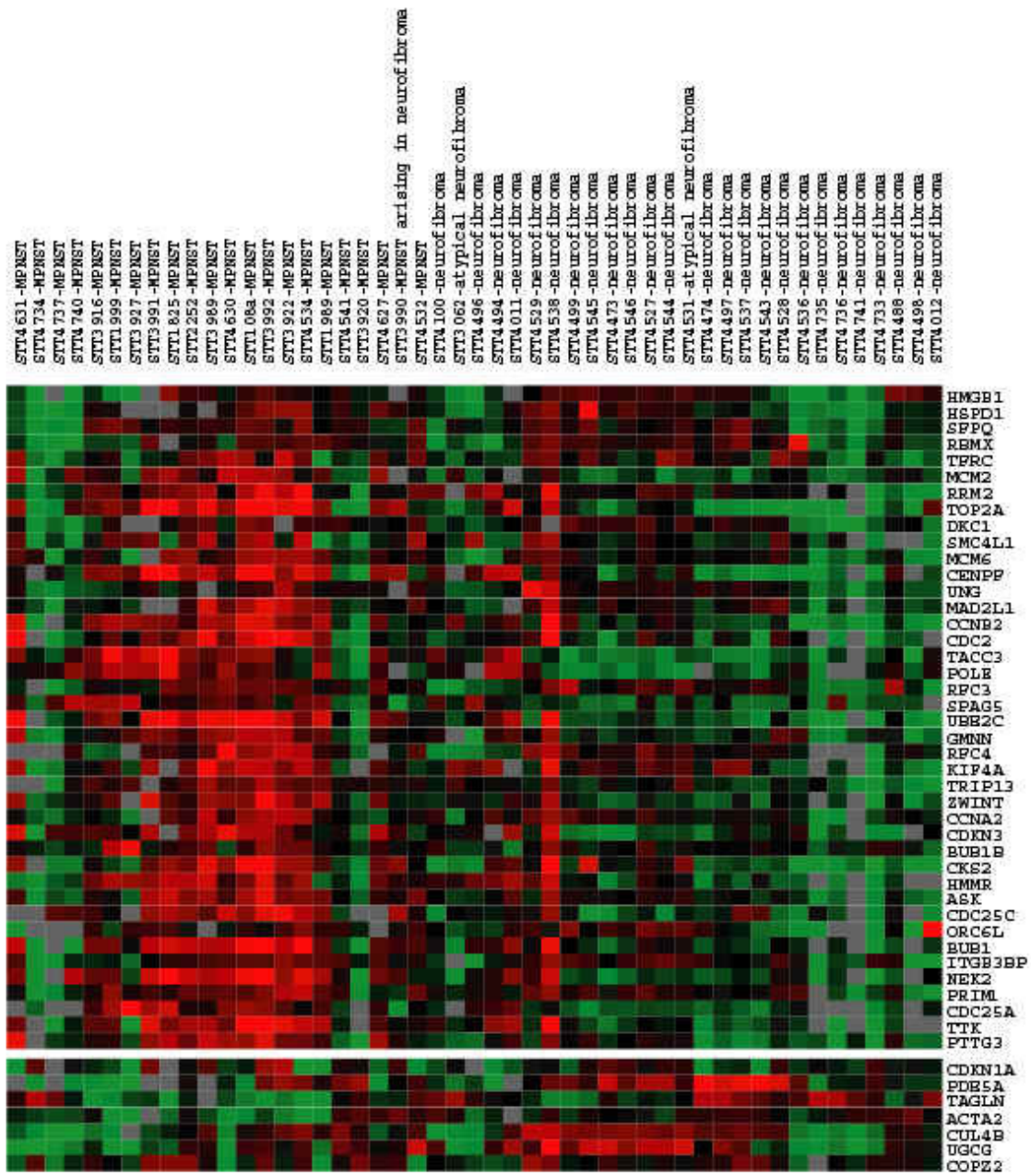


Figure 4

CLAC Plot for Sample: shgt157 aCGH:STT1999-MPNST||shgt157 ; (FDR=1.124)

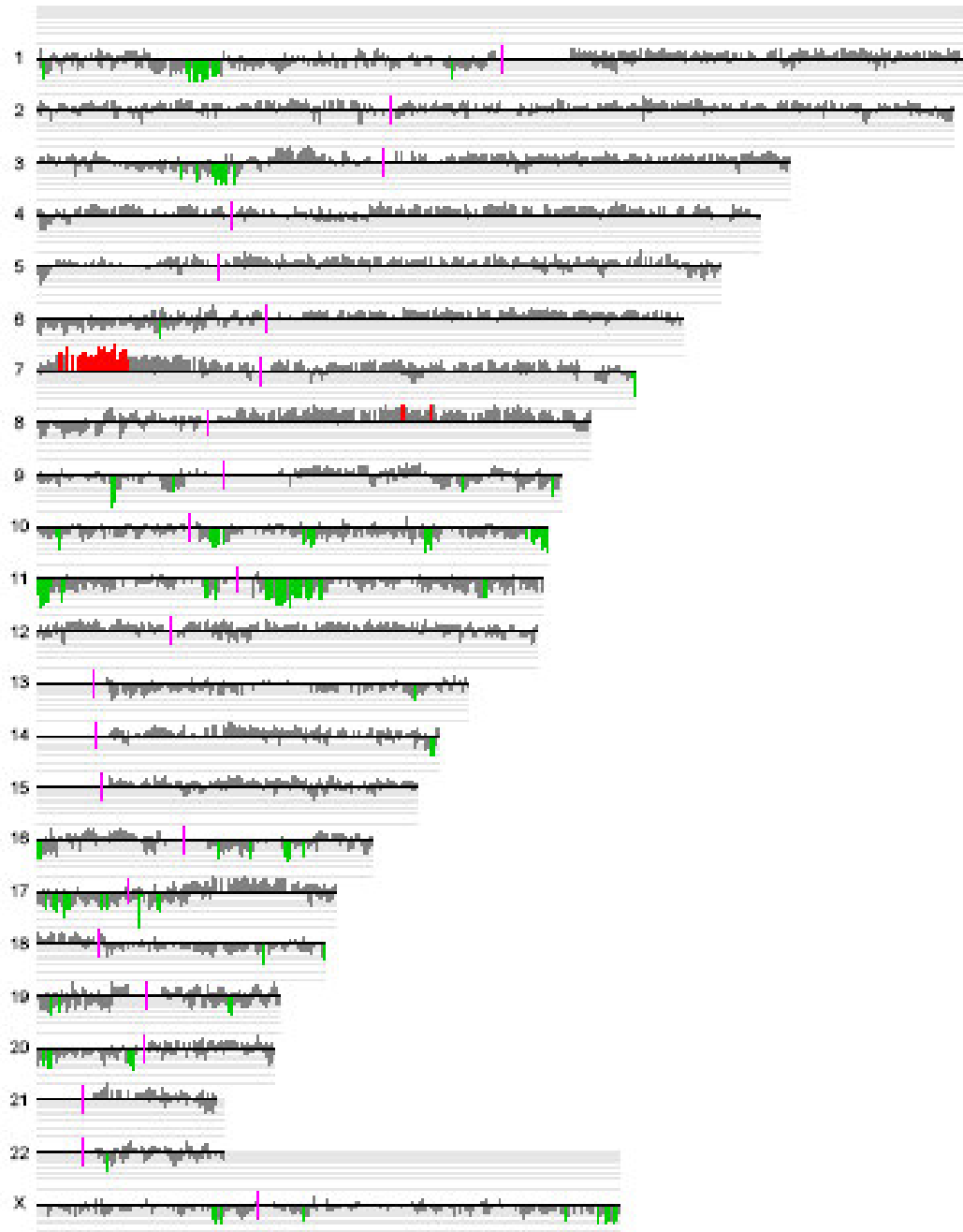


Figure 5

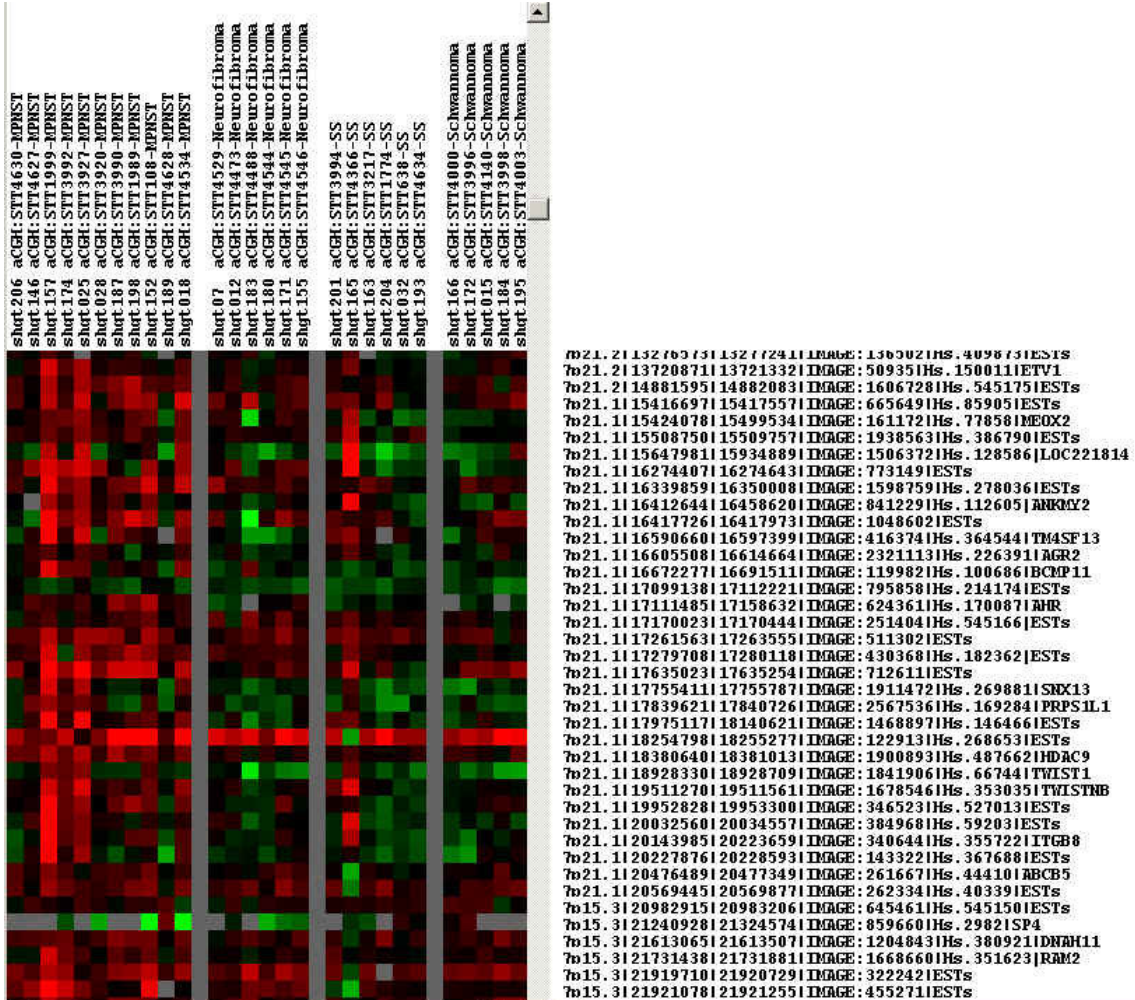


Figure 6

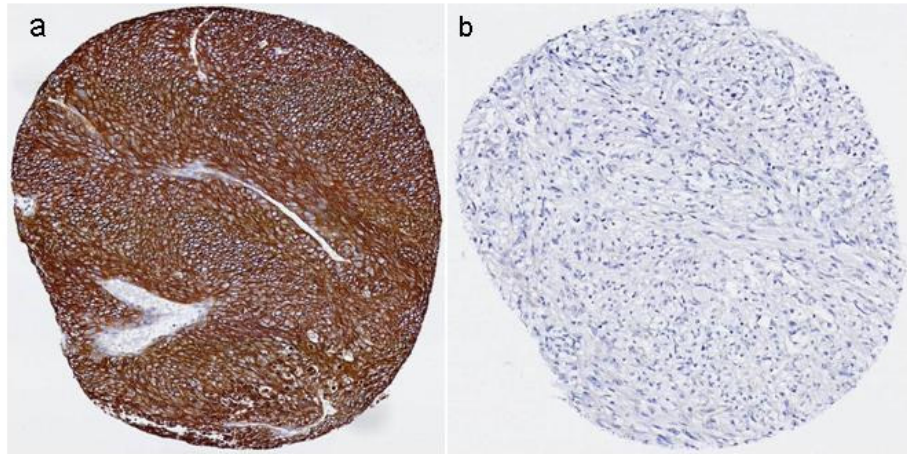


Table 1a

Gene ID	Gene Name	Score(d)	Numerator(r)	Denominator(s+s0)	Fold Change	q-value(%)
Transcribed locus Hs.563006 109176		1 2.834164779	1.96213277	0.692314288	1.74933517	0
MEST Mesoderm specific transcript homolog (mouse)		1 2.834070954	1.48733136	0.524803855	2.656708	0
SEMA3A Sema domain, immunoglobulin domain (Drosophila)		1 2.797788068	1.65449748	0.591359117	2.01760143	0
311247		1 2.795096772	1.58081866	0.565568491	3.65793119	0
DLK1 Delta-like 1 homolog (Drosophila) Hs.5337		1 2.740168449	2.4024471	0.876751611	3.99590244	0
GATA3 GATA binding protein 3 Hs.524134 101		1 2.735315709	1.42643888	0.521489666	1.65427509	0
EIF2B3 **Eukaryotic translation initiation factor 2B		1 2.72409874	1.95833734	0.71889367	1.79573195	0
CRABP1 Cellular retinoic acid binding protein 1 Hs.646		1 2.708004868	1.79335949	0.662243819	2.62360169	0
C7orf30 **Chromosome 7 open reading frame 30 Hs.90572		1 2.695964132	1.21754194	0.451616519	2.73870014	0
CRABP1 Cellular retinoic acid binding protein 1 Hs.646		1 2.678193258	1.95121059	0.728554815	2.62166022	0
CPA3 Carboxypeptidase A3 (mast cell) Hs.646 101		1 2.642648787	1.63811502	0.619876173	2.1206464	0
PTK7 **PTK7 protein tyrosine kinase 7 Hs.90572		1 2.624436801	1.3881871	0.528946666	2.25544653	0
MSX1 Msh homeo box homolog 1 (Drosophila) Hs.5337		1 2.595013901	1.5428	0.594524754	1.68495479	0
SEMA3A Sema domain, immunoglobulin domain (Drosophila)		1 2.579686609	1.48643065	0.5762059	2.1681315	0.155871
PCOLCE Procollagen C-endopeptidase enhancer 1 Hs.656 107804		1 2.553936477	1.3243862	0.518566618	2.33491019	0.155871
CDC25C Cell division cycle 25C Hs.656 107804		1 2.54241504	1.27106807	0.49994515	2.42517082	0.155871
TWIST1 Twist homolog 1 (acrocephalosyndactyly 1)		1 2.477659329	1.58447464	0.639504642	1.99273416	0.155871
NEK2 NIMA (never in mitosis gene a)-related kinase 2		1 2.453772274	1.38526487	0.564545003	2.78719512	0.263675
PYCR1 Pyroline-5-carboxylate reductase 1 Hs.4		1 2.450170238	1.26515914	0.516355607	2.68954056	0.263675
MAPK7 Mitogen-activated protein kinase 7 Hs.15		1 2.443673444	1.07468978	0.439784533	2.06086749	0.263675
LOXL2 Lysyl oxidase-like 2 Hs.116479 114413		1 2.42454404	1.08726804	0.448442271	2.3649708	0.263675
SOX4 SRY (sex determining region Y)-box 4 Hs.15		1 2.422517913	1.09339355	0.451345909	2.2274716	0.263675
PCOLCE Procollagen C-endopeptidase enhancer 1 Hs.656 107804		1 2.411383867	1.22883817	0.509598737	1.88517645	0.263675
KIAA0101 KIAA0101 Hs.81892 115900		1 2.399753154	1.3918055	0.57997861	2.52531365	0.263675
THBS4 Thrombospondin 4 Hs.211426 119951		1 2.367300866	1.34859946	0.569678101	3.0266392	0.331436
POSTN Periostin, osteoblast specific factor Hs.1		1 2.344295225	1.61655197	0.689568427	2.4372523	0.380451
DLG7 Discs, large homolog 7 (Drosophila) Hs.77		1 2.300111514	1.38762938	0.60328787	2.7864496	0.541556
UBE2C Ubiquitin-conjugating enzyme E2C Hs.93		1 2.294886553	1.28415726	0.559573307	3.29303302	0.541556
MRC2 Mannose receptor, C type 2 Hs.7835 11		1 2.246300766	1.03514487	0.460822028	1.97127092	0.62084
ADNP Activity-dependent neuroprotector Hs.2933		1 2.243387009	1.03119293	0.459658957	1.84716054	0.62084
LOC388152 Hypothetical protein FLJ90297 Hs.40		1 2.23629343	0.9725651	0.434900486	1.90720712	0.62084
TPX2 TPX2, microtubule-associated, homolog (Xeroderma pigmentosum)		1 2.220806951	1.40647577	0.633317439	2.49893165	0.62084
CTHRC1 Collagen triple helix repeat containing 1 Hs.5337		1 2.201981441	1.21440124	0.551503847	2.37237273	0.62084
DLK1 Delta-like 1 homolog (Drosophila) Hs.5337		1 2.185263084	1.88921587	0.864525597	3.95124394	0.771826
ELN Elastin (supravalvular aortic stenosis, Williams syndrome)		1 2.177267457	1.183165	0.543417389	2.25469337	0.771826
FLJ12505 Hypothetical protein FLJ12505 Hs.968		1 2.171623144	1.43649849	0.661486083	1.66080485	0.771826
CDKN3 Cyclin-dependent kinase inhibitor 3 (CDK2 regulatory subunit 3)		1 2.162243036	1.01874214	0.471150617	2.40635839	0.771826
IGF2BP2 Insulin-like growth factor binding protein 2		1 2.16132683	1.13202857	0.523765566	2.49249236	0.771826

Table 1b

Gene ID	Gene Name	Score(d)	Numerator(r)	Denominator(s+s0)	Fold Change	q-value(%)
TFPI Tissue factor pathway inhibitor (lipop	1	4.613441221	2.35827778	0.511175425	3.59386882	0
TFPI Tissue factor pathway inhibitor (lipop	1	4.229299539	2.19805787	0.519721493	3.00064105	0
PALMD Palmelphin Hs.483993 22526	1	4.178120688	2.2160006	0.530382141	5.1625263	0
ADH1B Alcohol dehydrogenase IB (class	1	4.134088114	2.59839167	0.628528371	3.6483497	0
PALMD Palmelphin Hs.483993 31483	1	3.940705439	1.97443651	0.501036309	4.58785848	0
PALMD Palmelphin Hs.483993 11056	1	3.763405196	2.18851058	0.581524037	5.65771928	0
ELOVL2 Elongation of very long chain fatt	1	3.734113866	2.18187784	0.584309403	5.3985426	0
DSCR1L1 Down syndrome critical region	1	3.717612014	1.8225	0.490234052	3.39972502	0
ABCG2 ATP-binding cassette, sub-family	1	3.687550758	1.61480913	0.437908312	3.09340554	0
LUM Lumican Hs.406475 102662	1	3.638690565	2.41116296	0.662645784	3.40779244	0
HSPC159 HSPC159 protein Hs.372208	1	3.558899335	1.60042407	0.449696359	3.27038842	0
LUM Lumican Hs.406475 110722	1	3.541719272	2.29288889	0.647394306	2.86473787	0
OMD Osteomodulin Hs.94070 114768	1	3.535697485	1.62297685	0.459025937	3.02783159	0
114871	1	3.509166045	2.16945556	0.618225392	3.47891429	0
IGF1 Insulin-like growth factor 1 (somatom	1	3.49570566	1.57951799	0.451845248	3.51097942	0
BCHE Butyrylcholinesterase Hs.420483	1	3.458963521	2.03993783	0.589754075	8.19084838	0
TGFBR2 Transforming growth factor, beta	1	3.438660607	1.97616204	0.574689469	3.56641801	0
SOX5 SRY (sex determining region Y)-bo	1	3.413685036	1.93384259	0.566497076	3.92246288	0
ARGBP2 Sorbin and SH3 domain contain	1	3.375596872	2.24805053	0.665971268	2.41323567	0
RASSF2 Ras association (RalGDS/AF-6)	1	3.363037447	1.51283333	0.449841358	2.6661658	0
MRE11A MRE11 meiotic recombination 1	1	3.360484562	1.45137484	0.431894512	2.55488429	0
TM4SF1 Transmembrane 4 L six family m	1	3.339574713	1.59829444	0.478592211	2.65659715	0
LAMA4 Laminin, alpha 4 Hs.213861 1	1	3.338171702	1.63975802	0.491214404	2.83557883	0
AOC3 Amine oxidase, copper containing	1	3.333153788	1.45409848	0.436253042	2.59675707	0
SIAT10 ST3 beta-galactoside alpha-2,3-si	1	3.326431076	2.04631728	0.615169002	3.4291595	0
DSCR1L1 Down syndrome critical region	1	3.300972344	1.61077672	0.487970377	2.83995666	0
APOD Apolipoprotein D Hs.522555 10	1	3.29272151	2.84420231	0.863784655	2.75957315	0
CYP1B1 Cytochrome P450, family 1, sub	1	3.286552521	2.38707407	0.726315511	3.82091006	0
ADD3 ADD3 protein Hs.44004 31700	1	3.276640796	1.46332407	0.446592765	2.75235183	0
THBD Thrombomodulin Hs.2030 9859	1	3.267356009	1.43309676	0.438610533	2.62612859	0
ADAMTS1 ADAM metalloproteinase with t	1	3.254769201	1.54590506	0.474966107	3.1826151	0
CYP1B1 Cytochrome P450, family 1, sub	1	3.249250477	1.91551667	0.589525701	3.52854608	0
APOD Apolipoprotein D Hs.522555 10	1	3.248499027	2.21792593	0.682754068	2.51354394	0
Transcribed locus Hs.247150 119666	1	3.246954485	1.91940741	0.591140842	2.96701383	0
TNFSF10 Tumor necrosis factor (ligand) s	1	3.245061059	1.58168342	0.48741253	2.69025679	0
Transcribed locus Hs.560535 107090	1	3.210248831	1.66014815	0.517140021	3.93922443	0
ALDH1A3 Aldehyde dehydrogenase 1 fam	1	3.191025506	1.79035185	0.561058459	3.65989277	0
RALGPS2 Ral GEF with PH domain and t	1	3.189402776	1.54962037	0.485865373	2.4588726	0
GPA33 Glycoprotein A33 (transmembran	1	3.178911735	1.48168071	0.466096839	3.39184294	0
CMAH Cytidine monophosphate-N-acetyl	1	3.177191844	1.47846759	0.465337841	2.70329248	0
SIAT10 ST3 beta-galactoside alpha-2,3-si	1	3.151065365	1.74836111	0.554847618	3.12654065	0
ADAMTS1 ADAM metalloproteinase with t	1	3.116508135	1.73102381	0.555436962	3.37394641	0
OSTM1 Osteopetrosis associated transm	1	3.097085332	1.33229558	0.430177226	2.32705214	0
STK17A Serine/threonine kinase 17a (apc	1	3.095482441	1.31496049	0.424799856	2.71128593	0
CFH Complement factor H Hs.363396	1	3.080627131	1.74108208	0.565171312	2.30371616	0

Table 1c

Row	Gene ID	Gene Nat	Score(d)	Numerator(r)	Denominator(s+s0)	Fold Change	q-value(%)
4704	SERPINA5 Serpin	1	6.004949059	3.72711249	0.620673456	15.4244079	0
4703	SERPINA5 Serpin	1	5.676090819	3.36553312	0.592931513	12.642694	0
5263	MAL Mal, T-cell dif	1	4.793000991	3.77839161	0.788314381	7.073874	0
329	SLC15A3 Solute c	1	4.768834072	2.43471204	0.510546605	4.92570351	0
4988	APOC2 Apolipopro	1	4.763704629	3.4394258	0.722006519	5.00166363	0
5264	Transcribed locus	1	4.609618204	3.3866573	0.734693667	5.28829885	0
5156	CTNNA1 Catenin	1	4.555031264	2.84420979	0.624410597	4.56085308	0
412	SLC2A5 Solute ca	1	4.478826003	3.09964216	0.692065768	4.83704532	0
4977	ANK3 Ankyrin 3, n	1	4.442590053	2.85613559	0.642898749	5.12824732	0
4987	APCS Amyloid P c	1	4.282614836	2.45367855	0.572939349	3.99470135	0
5265	PCOLN3 Procollag	1	4.204916561	2.85560315	0.679110538	5.3145688	0
4978	115174	1	4.201732093	2.5764347	0.613183954	4.59519355	0
4986	ALOX5AP Arachidi	1	4.180881601	2.74828105	0.657344865	4.22120786	0
411	HLA-DRB5 Major h	1	4.076986198	2.15998776	0.529800116	3.43376649	0
5261	LOC286191 Hypotl	1	4.044751599	2.55327209	0.631255598	4.67333867	0
5260	RPESP RPE-spon	1	4.001363666	2.25392033	0.563288048	4.10764883	0
5262	L1CAM L1 cell adh	1	3.995710183	3.16762448	0.792756314	4.71111161	0
404	HLA-DRB5 **Major	1	3.9708283	2.25247173	0.567254879	3.67641568	0
4979	ANK3 Ankyrin 3, n	1	3.95383411	2.55599524	0.64645991	4.55382756	0
414	RASSF4 Ras assoc	1	3.886845201	2.13090482	0.548235061	3.59405996	0
5134	SIAT7B ST6 (alpha	1	3.882142988	2.84759441	0.733510954	3.44583927	0
359	RAB20 RAB20, me	1	3.860581424	1.84690117	0.478399744	3.39837851	0
5114	SOX10 SRY (sex d	1	3.842512751	3.20823341	0.834931103	2.92161566	0
429	FEZ1 Fasciculation	1	3.841767155	1.78518963	0.464679288	3.13038672	0
5255	CRYAB Crystallin,	1	3.839845731	2.72711906	0.710215789	3.93775379	0
5259	FLJ14525 Hypothe	1	3.830032639	1.96455754	0.512934934	3.35821422	0
372	APOC1 Apolipopro	1	3.829958868	2.59238462	0.676870093	3.31288185	0
4701	CDH2 Cadherin 2,	1	3.819404209	2.30206056	0.60272766	4.08702312	0
4696	MAP1B Microtubul	1	3.803994797	1.89594246	0.498408269	3.27861955	0
386	MS4A7 Membrane	1	3.79206023	2.07331923	0.546752715	3.76365393	0
4705	C10orf45 Chromos	1	3.78417288	2.01348679	0.53208108	3.62430799	0
4976	SORCS1 Sortilin-re	1	3.778582031	2.80474522	0.742274535	3.23383023	0
389	VMD2 Vitelliform n	1	3.755890899	2.09299767	0.557257313	3.19643441	0
403	HLA-DRB1 Major h	1	3.749667044	2.0737798	0.553057051	3.15795577	0
4971	KCNMB4 Potassiu	1	3.745622326	2.26450155	0.604572847	4.23002216	0
1135	EPB41L4B Erythro	1	3.739550356	2.22678516	0.595468694	5.1477026	0
405	HLA-DRB1 Major h	1	3.721375543	2.28107366	0.612965188	2.96282595	0
488	MT1K Metallothion	1	3.708932185	1.93780342	0.522469358	4.25537481	0
5257	SPTBN1 Spectrin,	1	3.706913477	2.11531119	0.570639483	3.49559244	0
410	HLA-DRB1 Major h	1	3.704294151	1.93705393	0.522921196	3.30765227	0
371	APOC1 Apolipopro	1	3.699234789	2.48006488	0.670426459	3.11366049	0
4697	MS4A8B Membran	1	3.691777637	1.75859091	0.476353422	3.26621262	0
5115	SNCA Synuclein, a	1	3.691175466	2.87982867	0.78019284	3.5942503	0

Table 1d

Gene ID	Gene Name	Score(d)	Numerator(r)	Denominator(s+s0)	Fold Change	q-value(%)
SSX1 Synovial sarcoma, X breakpoint 1 Hs.43	1	7.587880343	3.99983325	0.527134465	11.4705629	0
ZIC2 Zic family member 2 (odd-paired homolog,	1	5.647026249	4.04358187	0.716055086	23.9644481	0
EFNB3 Ephrin-B3 Hs.26988 103884	1	5.148958369	3.33042946	0.646816156	7.79849705	0
SIX4 Sixe oculis homeobox homolog 4 (Drosoph	1	4.676161329	3.32675693	0.711429032	6.97722991	0
CRABP2 Cellular retinoic acid binding protein 2	1	4.63261851	3.45109148	0.744954817	6.511293	0
TGFB2 Transforming growth factor, beta 2 Hs.	1	4.50245711	3.05007999	0.677425663	6.30330369	0
CRABP2 Cellular retinoic acid binding protein 2	1	4.486542954	2.72729975	0.607884462	5.37323988	0
CA14 Carbonic anhydrase XIV Hs.528988 22	1	4.440070067	3.03882291	0.684408774	9.48300046	0
COL4A5 Collagen, type IV, alpha 5 (Alport synd	1	4.334618239	2.85740515	0.659205722	6.51321094	0
MSX2 Msh homeo box homolog 2 (Drosophila)	1	4.33384269	3.15208153	0.727317937	11.1305882	0
EGFL3 EGF-like-domain, multiple 3 Hs.56186	1	4.116669843	2.44159999	0.593100754	4.33070308	0
319147	1	4.086960689	2.68038958	0.655839334	8.62519096	0
106113	1	4.074052302	2.41630268	0.593095647	6.70830919	0
ENC1 Ectodermal-neural cortex (with BTB-like d	1	3.999189769	2.2361502	0.559150809	4.77580628	0
ENC1 Ectodermal-neural cortex (with BTB-like d	1	3.941565627	2.91637214	0.739901962	6.83013292	0
TLE1 Transducin-like enhancer of split 1 (E(sp1)	1	3.933089616	1.96836848	0.500463674	3.62235235	0
FZD1 Frizzled homolog 1 (Drosophila) Hs.942	1	3.900561186	2.61420493	0.670212516	5.47643838	0
WNT5A Wingless-type MMTV integration site fa	1	3.883754141	2.46655535	0.635095648	4.26568077	0
SHANK2 SH3 and multiple ankyrin repeat doma	1	3.872202089	2.45165092	0.633141262	6.19380079	0
MSX2 Msh homeo box homolog 2 (Drosophila)	1	3.839887634	2.90608712	0.756815666	8.396491	0
HSRG1 MON1 homolog B (yeast) Hs.513743	1	3.81923571	2.4376584	0.638258171	13.0023622	0
NXN **Nucleoredoxin Hs.527989 99548	1	3.814806952	2.32950986	0.610649474	4.74055278	0
EBPL Emopamil binding protein-like Hs.43327	1	3.768695546	1.78197505	0.472836033	3.14161052	0
TACSTD1 Tumor-associated calcium signal trar	1	3.748359159	3.02219536	0.806271552	26.4938469	0
TMEM30B Transmembrane protein 30B Hs.14	1	3.697434778	2.47869531	0.67038243	5.3271336	0
TLE4 Transducin-like enhancer of split 4 (E(sp1)	1	3.685344091	1.90286449	0.516332925	3.86286493	0
FOXD1 Forkhead box D1 Hs.519385 101053	1	3.673260968	2.28149261	0.621108227	4.26885681	0
MLLT3 Myeloid/lymphoid or mixed-lineage leuke	1	3.660170485	2.38412971	0.651371218	5.02421071	0
CRABP1 Cellular retinoic acid binding protein 1	1	3.649427519	3.21280794	0.880359434	4.124915	0
TACSTD1 Tumor-associated calcium signal trar	1	3.64715627	3.04224236	0.834140939	23.6065738	0
FOXC1 Forkhead box C1 Hs.348883 120223	1	3.63808051	1.83013909	0.50305074	3.28205873	0
COL27A1 Collagen, type XXVII, alpha 1 Hs.49	1	3.631422802	1.91954262	0.528592435	3.97011589	0
LOC152485 Hypothetical protein LOC152485 I	1	3.604161901	2.15574047	0.598125315	3.38293195	0
ZNF423 Zinc finger protein 423 Hs.530930 30	1	3.588203165	2.06748531	0.57618959	3.49184109	0
CRA Myotubularin related protein 11 Hs.42514	1	3.580370696	1.74973832	0.488703117	3.69298999	0
AUTS2 Autism susceptibility candidate 2 Hs.2	1	3.574285204	1.81889804	0.508884417	3.08987294	0
PRAME Preferentially expressed antigen in mel	1	3.567577233	1.8957265	0.531376442	2.89064963	0
ITM2C Integral membrane protein 2C Hs.1115	1	3.540345798	1.77246385	0.500647098	3.3639521	0
KIAA0888 KIAA0888 protein Hs.91662 1112	1	3.529635959	2.1879862	0.619890046	4.21348176	0
C1orf9 Chromosome 1 open reading frame 9 H	1	3.522497804	2.28997606	0.650100068	7.46953531	0
MTERF Mitochondrial transcription termination f	1	3.52186333	2.00955935	0.570595495	5.25364604	0
AXIN2 Axin 2 (conductin, axil) Hs.156527 30	1	3.516936933	1.90479454	0.541606113	3.06175147	0
MYO9B Myosin IXB Hs.123198 104290	1	3.489518058	2.07132559	0.593585005	4.47507892	0
FZD1 Frizzled homolog 1 (Drosophila) Hs.942	1	3.481759896	2.32872728	0.668836264	4.89941014	0
FGFR3 Fibroblast growth factor receptor 3 (ach	1	3.472690014	1.68659252	0.485673213	4.07241832	0

Table 2

Gene Set	SIZE	ES	NES	NOM p-value
SIG_PIP3_signaling_in_B_lymphocytes	36	0.539579	1.68401	0.035714
ST_Tumor_Necrosis_Factor_Pathway	27	0.533768	1.607012	0.025341
TGF_Beta_Signaling_Pathway	41	0.445959	1.518109	0.034417
cell_surface_receptor_linked_signal_transduction	73	0.428377	1.515348	0.069565
ST_JNK_MAPK_Pathway	49	0.483808	1.505573	0.016667
G13_Signaling_Pathway	26	0.510994	1.491692	0.009542
NFKB_INDUCED	91	0.39855	1.488427	0.068136
ST_Fas_Signaling_Pathway	45	0.386641	1.459184	0.014706
Met Pathway	51	0.473679	1.445063	0.078671

Table 3a

TGFB signaling pathway

PROBE	GENE SYMBOL	GENE_TITLE	RANK IN GENE LIST	CORE_ENRICHMENT
IMAGE:841149	TGFBR2	transforming growth factor, beta receptor II (70/80kDa)	14	Yes
IMAGE:824109	ZFH1B	zinc finger homeobox 1b	26	Yes
IMAGE:429042	THBS1	thrombospondin 1	673	Yes
IMAGE:364194	SKIL	SKI-like	777	Yes
IMAGE:767844	MADH2	MAD, mothers against decapentaplegic homolog 2 (Drosophila)	868	Yes
IMAGE:726035	JUN	v-jun sarcoma virus 17 oncogene homolog (avian)	1095	Yes
IMAGE:824193	TGFBR1	transforming growth factor, beta receptor I	1170	Yes
IMAGE:774754	CTNNB1	catenin (cadherin-associated protein), beta 1, 88kDa	1354	Yes
IMAGE:282838	ZFH1B	zinc finger homeobox 1b	1357	Yes
IMAGE:840691	STAT1	signal transducer and activator of transcription 1, 91kDa	1392	Yes
IMAGE:26474	FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog	1457	Yes
IMAGE:788421	MADH4	MAD, mothers against decapentaplegic homolog 4 (Drosophila)	1618	Yes
IMAGE:269425	ZFH1B	zinc finger homeobox 1b	1696	Yes
IMAGE:358531	JUN	v-jun sarcoma virus 17 oncogene homolog (avian)	1702	Yes
IMAGE:280356	MADH2	MAD, mothers against decapentaplegic homolog 2 (Drosophila)	1803	Yes
IMAGE:269269	TGFBR3	transforming growth factor, beta receptor III (betaglycan, 300kDa)	2188	Yes
IMAGE:23275	MADH4	MAD, mothers against decapentaplegic homolog 4 (Drosophila)	2366	Yes
IMAGE:545503	STAT1	signal transducer and activator of transcription 1, 91kDa	2501	Yes
IMAGE:1883559	FST	follicle-stimulating hormone receptor	2574	Yes

ST_Tumor_Necrosis_Factor_Pathway

PROBE	GENE SYMBOL	GENE_TITLE	RANK IN GENE LIST	CORE_ENRICHMENT
IMAGE:813671	HRB	HIV-1 Rev binding protein	60	Yes
IMAGE:813714	CFLAR	CASP8 and FADD-like apoptosis regulator	491	Yes
IMAGE:34852	BIRC2	baculoviral IAP repeat-containing 2	717	Yes
IMAGE:272632	NR2C2	nuclear receptor subfamily 2, group C, member 2	814	Yes
IMAGE:309776	CFLAR	CASP8 and FADD-like apoptosis regulator	850	Yes
IMAGE:299468	NR2C2	nuclear receptor subfamily 2, group C, member 2	893	Yes
IMAGE:51921	BAG4	BCL2-associated athanogene 4	981	Yes
IMAGE:726035	JUN	v-jun sarcoma virus 17 oncogene homolog (avian)	1095	Yes
IMAGE:279974	NR2C2	nuclear receptor subfamily 2, group C, member 2	1277	Yes
IMAGE:358531	JUN	v-jun sarcoma virus 17 oncogene homolog (avian)	1702	Yes
IMAGE:127032	BIRC3	baculoviral IAP repeat-containing 3	1853	Yes
IMAGE:2508563	TNFAIP3	tumor necrosis factor, alpha-induced protein 3	3080	Yes
IMAGE:796134	MAP3K7	mitogen-activated protein kinase kinase kinase 7	3648	Yes
IMAGE:843319	HRB	HIV-1 Rev binding protein	3789	Yes
IMAGE:429574	CASP3	caspase 3, apoptosis-related cysteine protease	3837	Yes

Table 3b**Cell_surface_receptor_linked_signal_transduction**

PROBE	GENE SYMBOL	GENE_TITLE	RANK IN GENE LIST	CORE_ENRICHMENT
IMAGE:49665	EDNRB	endothelin receptor type B	10	Yes
IMAGE:768246	PRKCA	protein kinase C, alpha	149	Yes
IMAGE:208718	ANXA1	annexin A1	203	Yes
IMAGE:768452	C21orf4	chromosome 21 open reading frame 4	264	Yes
IMAGE:503741	LIFR	leukemia inhibitory factor receptor	287	Yes
IMAGE:208001	CD59	CD59 antigen p18-20	305	Yes
IMAGE:782812	IFNAR1	interferon (alpha, beta and omega) receptor 1	423	Yes
IMAGE:204897	PLCG2	phospholipase C, gamma 2 (phosphatidylinositol-specific)	608	Yes
IMAGE:271050	EDNRB	endothelin receptor type B	657	Yes
IMAGE:897821	IL13RA1	interleukin 13 receptor, alpha 1	661	Yes
IMAGE:34852	BIRC2	baculoviral IAP repeat-containing 2	717	Yes
IMAGE:141931	BAIAP1	BAI1-associated protein 1	921	Yes
IMAGE:46694	LIFR	leukemia inhibitory factor receptor	1091	Yes
IMAGE:229365	PTPRC	protein tyrosine phosphatase, receptor type, C	1201	Yes
IMAGE:108837	CCL2	chemokine (C-C motif) ligand 2	1411	Yes
IMAGE:295889	LIFR,SHMT2	serine hydroxymethyltransferase 2 (mitochondrial)leukemia inhibitory factor receptor	1498	Yes
IMAGE:146671	IL1R1	interleukin 1 receptor, type I	1514	Yes
IMAGE:435470	PDGFA	platelet-derived growth factor alpha polypeptide	1518	Yes
IMAGE:841238	IL7R	interleukin 7 receptor	1685	Yes
IMAGE:2471879	PTPRC	protein tyrosine phosphatase, receptor type, C	1694	Yes
IMAGE:190887	MYD88	myeloid differentiation primary response gene (88)	1749	Yes
IMAGE:127032	BIRC3	baculoviral IAP repeat-containing 3	1853	Yes
IMAGE:768561	CCL2	chemokine (C-C motif) ligand 2	1871	Yes
IMAGE:2514377	BRD8	bromodomain containing 8	1907	Yes
IMAGE:785575	IFNGR2	interferon gamma receptor 2 (interferon gamma transducer 1) Homo sapiens mRNA; cDNA DKFZp779B1535 (from clone DKFZp779B1535)casein kinase 1, alpha 1	1916	Yes
IMAGE:298402	CSNK1A1	casein kinase 1, alpha 1	2115	Yes
IMAGE:704459	CD69	CD69 antigen (p60, early T-cell activation antigen)	2162	Yes
IMAGE:201890	BIRC3	baculoviral IAP repeat-containing 3	2229	Yes
IMAGE:501431	LIFR	leukemia inhibitory factor receptor	2262	Yes
IMAGE:753743	IL6ST	interleukin 6 signal transducer (gp130, oncostatin M receptor)	2346	Yes
IMAGE:428231	BIRC3	baculoviral IAP repeat-containing 3	2537	Yes
IMAGE:687592	OSMR	oncostatin M receptor	2559	Yes
IMAGE:433490	CSNK1A1	casein kinase 1, alpha 1	2657	Yes

Table 3c**JNK_MAPK_Pathway**

PROBE	GENE SYMBOL	GENE_TITLE	RANK IN GENE LIST	CORE_ENRICHMENT
IMAGE:199624	GAB1	GRB2-associated binding protein 1	19	Yes
IMAGE:593306	MAP3K1	mitogen-activated protein kinase kinase kinase 1 mitogen-activated protein kinase kinase kinase 7	47	Yes
IMAGE:838692	MAP3K7IP2	interacting protein 2	501	Yes
IMAGE:810230	MAP3K1	mitogen-activated protein kinase kinase kinase 1	655	Yes
IMAGE:119133	MAPK8	mitogen-activated protein kinase 8	711	Yes
IMAGE:272632	NR2C2	nuclear receptor subfamily 2, group C, member 2	814	Yes
IMAGE:726035	JUN	v-jun sarcoma virus 17 oncogene homolog (avian)	1095	Yes
IMAGE:279974	NR2C2	nuclear receptor subfamily 2, group C, member 2	1277	Yes
IMAGE:140337	PAPPA	pregnancy-associated plasma protein A	1373	Yes
IMAGE:1565455	NR2C2	nuclear receptor subfamily 2, group C, member 2	1382	Yes
IMAGE:588550	TRAF6	TNF receptor-associated factor 6	1478	Yes
IMAGE:146671	IL1R1	interleukin 1 receptor, type I	1514	Yes
IMAGE:50765	ATF2	activating transcription factor 2	1538	Yes
IMAGE:176565	MAP3K2	mitogen-activated protein kinase kinase kinase 2	1616	Yes
IMAGE:358531	JUN	v-jun sarcoma virus 17 oncogene homolog (avian)	1702	Yes
IMAGE:854746	CDC42	cell division cycle 42 (GTP binding protein, 25kDa)	1728	Yes
IMAGE:41333	MAP3K2	mitogen-activated protein kinase kinase kinase 2	1780	Yes
IMAGE:1880757	MAP3K5	mitogen-activated protein kinase kinase kinase 5	1983	Yes
IMAGE:1470508	MAPK8	mitogen-activated protein kinase 8	2203	Yes
IMAGE:123087	PAPPA	pregnancy-associated plasma protein A	2347	Yes
IMAGE:813648	DLD	dihydrolipoamide dehydrogenase	2534	Yes
IMAGE:365531	CDC42	cell division cycle 42 (GTP binding protein, 25kDa)	2569	Yes
IMAGE:129112	PAPPA	pregnancy-associated plasma protein A	2812	Yes

Table 3d

NFKB_induced signaling pathway

PROBE	GENE SYMBOL	GENE_TITLE	RANK IN GENE LIST	CORE_ENRICHMENT
IMAGE:25154	PLAT	suppressor of <i>S. cerevisiae</i> gcr2plasminogen activator, tissue	48	Yes
IMAGE:2545220	RABEP1	rabaptin, RAB GTPase binding effector protein 1	97	Yes
IMAGE:261246	SPTBN1	spectrin, beta, non-erythrocytic 1	107	Yes
IMAGE:280444	KIAA0608	KIAA0608 protein	247	Yes
IMAGE:701112	XPC	xeroderma pigmentosum, complementation group C	485	Yes
IMAGE:813714	CFLAR	CASP8 and FADD-like apoptosis regulator	491	Yes
IMAGE:489729	ETS1	v-ets erythroblastosis virus E26 oncogene homolog 1 (avian)	548	Yes
IMAGE:840708	SOD2	superoxide dismutase 2, mitochondrial	653	Yes
IMAGE:841332	ARHGDIB	Rho GDP dissociation inhibitor (GDI) beta	671	Yes
IMAGE:78148	SOD2	superoxide dismutase 2, mitochondrial	792	Yes
IMAGE:530185	CD83	CD83 antigen (activated B lymphocytes, immunoglobulin superfamily)	839	Yes
IMAGE:309776	CFLAR	CASP8 and FADD-like apoptosis regulator	850	Yes
IMAGE:857002	PNRC1	proline-rich nuclear receptor coactivator 1	985	Yes
IMAGE:2371739	SPTBN1	spectrin, beta, non-erythrocytic 1	1023	Yes
IMAGE:727251	CD9	CD9 antigen (p24)	1033	Yes
IMAGE:712559	SEC24A	SEC24 related gene family, member A (<i>S. cerevisiae</i>)	1209	Yes
IMAGE:108837	CCL2	chemokine (C-C motif) ligand 2	1411	Yes
IMAGE:784593	ARHE	ras homolog gene family, member E	1653	Yes
IMAGE:841238	IL7R	interleukin 7 receptor	1685	Yes
IMAGE:240651	UTX	ubiquitously transcribed tetratricopeptide repeat gene, X chromosome	1724	Yes
IMAGE:296556	FN1	fibronectin 1	1834	Yes
IMAGE:768561	CCL2	chemokine (C-C motif) ligand 2	1871	Yes
IMAGE:137296	SEC24A	SEC24 related gene family, member A (<i>S. cerevisiae</i>)	1938	Yes
IMAGE:321816	TNFAIP2	tumor necrosis factor, alpha-induced protein 2	1969	Yes
IMAGE:324383	FGF2	fibroblast growth factor 2 (basic)	2159	Yes
IMAGE:841008	GBP1	guanylate binding protein 1, interferon-inducible, 67kDa	2340	Yes
IMAGE:753587	BTN3A3	butyrophilin, subfamily 3, member A3	2398	Yes
IMAGE:489025	RAC1	ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1)	2438	Yes
IMAGE:415851	ARHE	ras homolog gene family, member E	2586	Yes
IMAGE:898332	NPR1	natriuretic peptide receptor A/guanylate cyclase A (atrionatriuretic peptide receptor A)	2832	Yes
IMAGE:878449	MAP1B	microtubule-associated protein 1B	2923	Yes
IMAGE:2404494	SEC24A	SEC24 related gene family, member A (<i>S. cerevisiae</i>)	3100	Yes
IMAGE:357031	TNFAIP6	tumor necrosis factor, alpha-induced protein 6	3117	Yes
IMAGE:768316	ABCC1	ATP-binding cassette, sub-family C (CFTR/MRP), member 1 chromosome condensation 1-like	3141	Yes
IMAGE:664121	LITAF	lipopolysaccharide-induced TNF factor	3218	Yes
IMAGE:346860	SOD2	superoxide dismutase 2, mitochondrial	3283	Yes
IMAGE:813614	SPRR1B	small proline-rich protein 1B (cornifin)	3488	Yes
IMAGE:186132	SELE	selectin E (endothelial adhesion molecule 1)	3504	Yes

Table 3e

Met Pathway

PROBE	GENE SYMBOL	GENE_TITLE	RANK IN GENE LIST	CORE_ENRICHMENT
IMAGE:199624	GAB1	GRB2-associated binding protein 1 phosphatase and tensin homolog (mutated in multiple advanced cancers 1)	19	Yes
IMAGE:1925760	PTEN	phosphatase and tensin homolog (mutated in multiple advanced cancers 1)	65	Yes
IMAGE:704905	RAP1A	RAP1A, member of RAS oncogene family protein tyrosine phosphatase, non-receptor type 11 (Noonan syndrome 1)	221	Yes
IMAGE:814776	PTPN11	phosphatase and tensin homolog (mutated in multiple advanced cancers 1)	331	Yes
IMAGE:366966	PTEN	phosphatase and tensin homolog (mutated in multiple advanced cancers 1)	345	Yes
IMAGE:502527	ITGA1	integrin, alpha 1 phosphoinositide-3-kinase, catalytic, alpha polypeptide	454	Yes
IMAGE:345430	PIK3CA	mitogen-activated protein kinase 8	600	Yes
IMAGE:119133	MAPK8	PTK2 protein tyrosine kinase 2	711	Yes
IMAGE:724892	PTK2	RAS p21 protein activator (GTPase activating protein) 1Homo sapiens mRNA; cDNA DKFZp566M223 (from clone DKFZp566M223)	1007	Yes
IMAGE:276639	Hs.350524,RASA1	v-jun sarcoma virus 17 oncogene homolog (avian)	1009	Yes
IMAGE:726035	JUN	met proto-oncogene (hepatocyte growth factor receptor)	1095	Yes
IMAGE:626841	MET	v-fos FBJ murine osteosarcoma viral oncogene homolog	1207	Yes
IMAGE:26474	FOS	mitogen-activated protein kinase 1	1457	Yes
IMAGE:970590	MAPK1	integrin, alpha 1	1536	Yes
IMAGE:785530	ITGA1	v-jun sarcoma virus 17 oncogene homolog (avian)	1667	Yes
IMAGE:358531	JUN	phosphatase and tensin homolog (mutated in multiple advanced cancers 1)	1702	Yes
IMAGE:322160	PTEN	son of sevenless homolog 1 (Drosophila)	1827	Yes
IMAGE:452423	SOS1	RAP1B, member of RAS oncogene family integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	1950	Yes
IMAGE:897961	RAP1B	integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	2029	Yes
IMAGE:325953	ITGB1	mitogen-activated protein kinase 8	2063	Yes
IMAGE:1470508	MAPK8	protein tyrosine phosphatase, non-receptor type 11 (Noonan syndrome 1)	2203	Yes
IMAGE:34773	PTPN11	phosphoinositide-3-kinase, regulatory subunit, polypeptide 1 (p85 alpha)	2269	Yes
IMAGE:39808	PIK3R1	integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	2503	Yes
IMAGE:343072	ITGB1	integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	2560	Yes
IMAGE:321189	RAP1B	RAP1B, member of RAS oncogene family	2568	Yes

APPENDICES

1. AACR 2006 abstract
2. Copy of manuscript in press in The American Journal of Surgical Pathology.

American Association for Cancer Research (AACR) 2006 annual meeting abstract.

Genome-wide transcriptome analysis of nerve sheath tumors

Subbaya Subramanian¹, Robert B. West¹, Torsten O. Nielsen², Brian P. Rubin³, Erinn Downs-Kelly⁴, John R. Goldblum⁴, Shirley Zhu¹, Kelli Montgomery¹, Pancras C.W Hogendoorn⁵, Christopher L. Corless⁶, Andre M. Oliveira⁷, Christopher D.M. Fletcher⁸, Matt van de Rijn¹

¹Department of Pathology, Stanford University Medical Center, Stanford, California, USA;

²Department of Pathology, Vancouver General Hospital, Vancouver, British Columbia, Canada;

³Department of Anatomic Pathology, University of Washington Medical Center, Seattle, Washington, USA;

⁴Department of Anatomic Pathology, Cleveland Clinic Foundation, Cleveland, Ohio, USA;

⁵Department of Pathology, Leiden University Medical Center, The Netherlands;

⁶Department of Pathology, Oregon Health and Science University, Portland, Oregon, USA;

⁷Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, Minnesota, USA;

⁸Department of Pathology, Brigham and Women's hospital and Harvard Medical School, Boston, Massachusetts, USA.

Malignant peripheral nerve sheath tumors (MPNSTs) are aggressive soft tissue tumors that arise from neurofibromas in patients with neurofibromatosis but also occur sporadically. Malignant transformation is a life threatening complication in patients with neurofibromatosis. The transformation of neurofibromas into MPNSTs is not completely understood at a molecular level. The goal of this study is to identify genes that will serve as molecular markers for progression of neurofibroma to MPNST, and to identify novel potential therapeutic targets. Gene expression profiling using 43000 spot cDNA microarrays was performed on 32 cases of MPNSTs, 23 schwannomas, 20 neurofibromas and 16 synovial sarcomas. Using unsupervised hierarchical clustering, most tumors grouped together according to tumor type. There appear to be at least two subtypes of MPNSTs. Analysis employing 'significance analysis of microarrays' identified genes that were differentially expressed in various nerve sheath tumors. A major trend in transformation from neurofibroma towards MPNST is accompanied by the loss of expression of a large number of genes, rather than widespread *de novo* increase in gene expression upon transformation. *NF1* is one of the genes for which loss of expression was noticed in most of the MPNSTs. Potential signaling pathways were identified using 'Gene Set Enrichment Analysis' method. The expression of genes associated with MET and TFGF signaling pathways in the majority of neurofibromas, but not in MPNST, suggest that these pathways are affected during malignant transformation. A detailed list of genes and the signaling pathways that are associated with various nerve sheath tumors will be discussed.

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Corresponding Author: Dr. Torsten O. Nielsen, MD/PhD

Corresponding Author's Institution: University of British Columbia

First Author: Jefferson Terry, MD

Order of Authors: Jefferson Terry, MD; Tsuyoshi Saito, MD/PhD; Subbaya Subramanian, PhD; Cindy Ruttan, MSc; Cristina R Antonescu, MD; John R Goldblum, MD; Erinn Downs-Kelly, MD; Christopher L Corless, MD/PhD; Brian P Rubin, MD/PhD; Matt van de Rijn, MD/PhD; Marc Ladanyi, MD; Torsten O Nielsen, MD/PhD

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Abstract: Synovial sarcoma is a soft tissue malignancy defined by the SYT-SSX fusion oncogene. Demonstration of the t(X;18) by cytogenetics, FISH or RT-PCR has become the gold standard for diagnosis, but practical considerations limit the availability of these methods. Gene expression profiling studies performed by several independent groups have consistently identified TLE1 as an excellent discriminator of synovial sarcoma from other sarcomas, including histologically similar tumors such as malignant peripheral nerve sheath tumor. TLE proteins (human homologues of groucho) are transcriptional corepressors that inhibit Wnt signaling and other cell fate determination signals, and so have an established role in repressing differentiation. We examined the expression of TLE proteins in synovial sarcoma and in a broad range of mesenchymal tumors using tissue microarrays to assess the value of anti-TLE antibodies in the

immunohistochemical confirmation of synovial sarcoma. We demonstrate that TLE expression is a consistent feature of synovial sarcoma using both a well-characterized monoclonal antibody recognizing the TLE family of proteins and a commercially available polyclonal antibody raised against TLE1. Both antibodies gave intense and/or diffuse nuclear staining in 91/94 molecularly-confirmed synovial sarcomas. Moderate staining is occasionally seen in schwannoma and solitary fibrous tumor/hemangiopericytoma. In contrast, TLE staining is detected much less frequently and at lower levels, if at all, in 40 other mesenchymal tumors. Our findings establish TLE as a robust immunohistochemical marker for synovial sarcoma, and may have implications for understanding the biology of synovial sarcoma and for developing experimental therapies for this cancer.

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Authors: Jefferson Terry MD¹, Tsuyoshi Saito MD/PhD², Subbaya Subramanian PhD³, Cindy Ruttan BSc¹, Cristina R Antonescu MD², John R Goldblum MD⁴, Erinn Downs-Kelly MD⁴, Christopher L Corless MD/PhD⁵, Brian P Rubin MD/PhD⁶, Matt van de Rijn MD/PhD³, Marc Ladanyi MD^{2†}, Torsten O Nielsen MD/PhD^{1†}

¹Genetic Pathology Evaluation Centre, British Columbia Cancer Agency, 600 West 10th Avenue, Vancouver, BC, Canada V5Z 4E6; ²Department of Pathology, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021; ³Department of Pathology, Stanford University Medical Center, 300 Pasteur Drive, Stanford, CA 94305; ⁴Department of Anatomic Pathology, Cleveland Clinic, 9500 Euclid Avenue, Cleveland, OH 44195; ⁵Department of Pathology and OHSU Cancer Institute, Oregon Health and Science University, Portland, OR 97239-3098; ⁶Department of Anatomical Pathology, University of Washington Medical Center, Seattle, WA 98195

† Corresponding authors

Addresses for correspondence and reprint requests:

Torsten O. Nielsen, Anatomical Pathology, Vancouver Coastal Health Research Institute, Room JP1502, 855 West 12th Avenue, Vancouver, British Columbia, Canada, V5Z 1M9. E-mail: torsten@interchange.ubc.ca.
Marc Ladanyi, Department of Pathology, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021. Phone: 212-639-6369; Fax: 212-717-3515; E-mail: ladanyim@mskcc.org.

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Abstract

Synovial sarcoma is a soft tissue malignancy defined by the *SYT-SSX* fusion oncogene. Demonstration of the t(X;18) by cytogenetics, FISH or RT-PCR has become the gold standard for diagnosis, but practical considerations limit the availability of these methods. Gene expression profiling studies performed by several independent groups have consistently identified *TLE1* as an excellent discriminator of synovial sarcoma from other sarcomas, including histologically similar tumors such as malignant peripheral nerve sheath tumor. TLE proteins (human homologues of *groucho*) are transcriptional corepressors that inhibit Wnt signaling and other cell fate determination signals, and so have an established role in repressing differentiation. We examined the expression of TLE proteins in synovial sarcoma and in a broad range of mesenchymal tumors using tissue microarrays to assess the value of anti-TLE antibodies in the immunohistochemical confirmation of synovial sarcoma. We demonstrate that TLE expression is a consistent feature of synovial sarcoma using both a well-characterized monoclonal antibody recognizing the TLE family of proteins and a commercially available polyclonal antibody raised against TLE1. Both antibodies gave intense and/or diffuse nuclear staining in 91/94 molecularly-confirmed synovial sarcomas. Moderate staining is occasionally seen in schwannoma and solitary fibrous tumor/hemangiopericytoma. In contrast, TLE staining is detected much less frequently and at lower levels, if at all, in 40 other mesenchymal tumors. Our findings establish TLE as a robust immunohistochemical marker for synovial sarcoma, and may have implications for understanding the biology of synovial sarcoma and for developing experimental therapies for this cancer.

Key words: Synovial sarcoma, TLE, immunohistochemistry, microarray, expression profiling

Introduction

Synovial sarcoma is a soft tissue malignancy typically occurring in the limbs of young adults, although it may arise at almost any age and anatomic location (10, 11). Synovial sarcomas can be segregated into three histologic subtypes: monophasic, (the most common form, composed entirely of spindle cells), biphasic (displaying both spindle cells and glandular-appearing epithelial components) and poorly differentiated synovial sarcoma (sheets of atypical small blue cells). Morphological variants can also be identified, such as calcifying and fibrous (11), widening the range of appearances and the differential diagnosis. Demonstration in an appropriate histologic context of t(X;18) by cytogenetics, fluorescence *in-situ* hybridization (FISH) or reverse-transcriptase polymerase chain reaction (RT-PCR) is considered the gold standard for the diagnosis of synovial sarcoma; however, several practical issues, including cost and the need for specialized equipment and personnel have limited the use of such diagnostic tests (5, 8). Correct diagnosis of synovial sarcoma based on histology alone can be challenging especially in small biopsies, as monophasic synovial sarcomas can appear similar to other spindle cell tumors (including malignant peripheral nerve sheath tumor (MPNST), fibrosarcoma and hemangiopericytoma), and poorly differentiated synovial sarcomas can resemble several tumor types including Ewing sarcoma. Immunoreactivity for epithelial markers such as cytokeratin and epithelial membrane antigen (EMA) is frequently used to aid in differentiating synovial sarcoma from other spindle cell neoplasms; however, these markers not only lack specificity (12, 27), but also are limited in sensitivity because such epithelial markers are only focally expressed in many synovial sarcomas and are completely negative in a subset of monophasic cases.

The t(X;18) translocation most commonly fuses either the *SSX1* or *SSX2* gene on chromosome X to the *SYT* gene on chromosome 18, resulting in the production of an SYT-SSX fusion protein. The function of SYT-SSX has yet to be fully defined, but combines transcriptional activation (*SYT*) and repression (*SSX*) domains and likely drives synovial sarcoma development through dysregulation of gene expression (10, 19). DNA microarray expression profiling, using different platforms, comparison groups and informatics approaches, has consistently shown a major association of the Wnt signaling pathway with synovial sarcoma (1, 3, 14, 20, 21, 25, 29). One prominent gene related to the Wnt pathway is *TLE1*, which has been found to be a good discriminator of synovial sarcoma in multiple studies (Table 1) (3, 20, 23, 29). *TLE1* is one of four *TLE* (Transducin-Like Enhancer of split) genes that encode human transcriptional repressors homologous to the *Drosophila* corepressor *groucho* (30); differential overexpression of *TLE2*, 3 and 4 has also been demonstrated in synovial sarcoma (1, 3, 25). *TLE* proteins are temporally expressed in embryogenesis where they are involved in developmental processes including neurogenesis, body patterning and hematopoiesis (6, 30, 31, 34). The repressive effect of Groucho and *TLE1* is dependent on phosphorylation status and involves histone deacetylase (HDAC) activity (4, 7, 16, 26, 35). The HDAC inhibitor FK228 has recently been shown to inhibit proliferation of synovial sarcoma, supporting the idea that *TLE1* overexpression may play an important role in synovial sarcoma pathobiology and identifying *TLE1* as a potential therapeutic target (17, 18).

The specificity of *TLE1* gene expression in synovial sarcoma, particularly when compared to other sarcomas, suggests that TLE1 may be clinically exploitable as an immunohistochemical marker. Here we investigate the protein expression of TLE in synovial sarcoma and in a broad range of mesenchymal neoplasms using tissue microarrays, to assess the value of TLE as a diagnostic marker for this sarcoma.

Materials & Methods

Tumor Samples and Tissue Microarrays

Tissue samples were retrieved from the archives of the Vancouver General Hospital (Vancouver, Canada), Stanford Medical Center (Stanford, CA), University of Washington (Seattle, WA), Cleveland Clinic (Cleveland, OH), Oregon Health & Science University (Portland, OR), and Memorial Sloan-Kettering Cancer Center (MSKCC, New York, NY). Slides corresponding to each archival sample were reviewed by at least two staff pathologists with subspecialty expertise in bone and soft tissue tumors, and representative areas in each original tissue block identified. The TA-19 synovial sarcoma tissue microarray has been previously described (24) and contains 44 molecularly-confirmed synovial sarcomas and 29 other sarcomas with related histologies. The MSKCC synovial sarcoma tissue microarrays contained 52 molecularly-confirmed synovial sarcomas each represented by one 3 mm and one 1 mm core (on different arrays). The TA-138 tissue microarray contains 44 cases of NF-1 related MPNST, 24 sporadic MPNST, 15 synovial sarcomas, 8 localized neurofibroma, 24 plexiform neurofibroma, 11 diffuse neurofibroma, 7 cellular schwannoma, 15 typical schwannoma, 4 perineurioma, 10 melanoma, 5 clear cell carcinoma, and 5 cases of dermatofibrosarcoma protuberans with fibrosarcomatous change. The sarcoma tissue microarrays TA-34 and TA-35 contain 421 benign and malignant soft tissue tumor specimens representing over 50 diagnostic entities and have been previously described (33), as has tissue microarray 03-008, which contains 121 cases of chondroid and osseous tumors (22). For each of these, a tissue microarrayer (Beecher Instruments, MD, USA) was used to extract duplicate 0.6 mm or 1.0 mm cores from representative areas of each original formalin-fixed paraffin-embedded tissue block and to transfer to the recipient microarray block, except for one of the MSKCC tissue microarrays for which 3 mm cores were obtained using a manual punch instrument. All tissue samples were collected according to protocols approved by the ethics committees at the contributing institutions.

Molecular confirmation of synovial sarcoma cases

The presence of t(X;18) in the synovial sarcoma cores and absence of t(X;18) in the non-synovial sarcoma cores with positive TLE staining in the TA-138, 03-008, TA-34 and TA-39 microarrays were verified using a previously described *SYT* breakapart probe FISH method (32). Briefly, a 6 μ m section was deparaffinized followed by demasking. Differentially labelled DNA BAC probes were hybridized to the samples overnight and the nuclei counterstained. The TA-19 synovial sarcoma microarray has been previously assessed in this manner (32). Synovial sarcoma cases in which the presence of t(X;18) could not be verified were removed. Cases on the MSKCC tissue microarrays were individually validated by *SYT*-*SSX* RT-PCR on frozen or

formalin fixed paraffin embedded material, as described previously (2), and included 35 SYT-SSX1 cases and 17 SYT-SSX2 cases.

Immunohistochemistry

Heat-induced epitope retrieval was performed by heating 4 μ m sections of each tissue microarray for 30–40 minutes in 10 mM EDTA buffer pH 8. Monoclonal rat anti-human pan-TLE antibody, which recognizes the highly conserved WD-40 domain, has been previously characterized (30) and was graciously provided by S. Stifani (Montreal Neurological Institute, Montréal, Canada). Polyclonal rabbit anti-TLE1 (M-101), which also cross-reacts to a lesser extent with TLE2, 3 and 4, was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Each microarray section was hybridized with a 1:2 dilution of monoclonal anti-pan-TLE or a 1:20 dilution of M-101 polyclonal anti-TLE1 using a Ventana automated immunostainer (Ventana, AZ) for 30 minutes followed by washing and hybridization with a 1:1000 dilution of HRP-conjugated anti-rat IgG antibody (Abcam, Cambridge, UK). M-101 staining was for comparative purposes also performed manually, using a 1:200 dilution and 4°C overnight incubation. Endogenous peroxidase activity was quenched and antibody visualized by incubation with 3,3'-diaminobenzidine for 10 minutes. TLE immunostaining was graded as “3+” (strong) if greater than 50% of tumor cells per core exhibited intense nuclear staining visible with a 4x low power objective lens, “2+” (moderate) if 10%–50% exhibited intense nuclear staining obvious at low power or greater than 50% nuclear staining well above background when assessed with 10x objective magnification, “1+” (weak) if less than 50% of cells exhibited weak to moderate nuclear staining and “0” (negative) for no visible nuclear staining. Where scores of duplicate cores were discrepant, the higher score was used. Uninterpretable sets of duplicate cores (i.e. no tumor cells, absence of viable cells or folded/lost tissue core) were excluded from analysis. Tumors with a score of 2+ or 3+ on at least one examined tissue microarray core were considered positive for TLE. Sections of each microarray were also stained with hematoxylin and eosin (H&E) using standard methods as an additional histologic reference.

Digital Images

Digital images of immunostained and H&E stained microarrays were acquired using a BLISS imager (Bacus Laboratories, Lombard, IL, USA). A relational database was constructed that correlates scoring and identification information with images of each core. This information is publicly accessible at <https://www.gpecimage.ubc.ca/tma/web/viewer.php>.

Results

TLE as an immunohistochemical marker

The consistent identification of strong *TLE* expression in synovial sarcoma, from several gene expression profiling studies in different laboratories using different DNA microarray platforms (Table 1) led us to investigate its value as a diagnostic immunohistochemical marker. Two antibodies against TLE1, a monoclonal antibody recognizing an epitope in the C-terminal WD-40 domain, previously shown to work in immunohistochemical applications (30), and a commercially-available polyclonal antibody raised against TLE1, were tested against 693 cases of adult soft tissue tumors including 94 molecularly-validated synovial sarcomas using a tissue microarray format.

Both antibodies gave intense, easy-to-interpret nuclear staining in positive cases. Examples of the four grades of staining, as described in materials and methods, are presented in Figure 1. Original images of tissue cores are available for public review at <https://www.gpecimage.ubc.ca/tma/web/viewer.php>, and includes H&E, pan-TLE monoclonal and M101 TLE1 polyclonal immunostains on the same (TA-138) tissue microarray for comparative purposes.

The two antibodies (monoclonal anti-pan-TLE and M101 polyclonal anti-TLE1) were tested on sequential sections of the TA-138 tissue microarray and found to be almost equivalent (8 discrepancies among 177 cases, Kappa statistic 0.78, $p < 10^{-25}$). Overall, the intensity of optimized staining with M101 was slightly less than with the monoclonal anti-pan-TLE; all discrepancies in scoring (two MPNST, 2 solitary fibrous tumor, 1 schwannoma and 3 synovial sarcomas) between the two antibodies were cases scored positive by pan-TLE and negative by M101. For rigour, tissue microarrays assessing antibody specificity among various sarcomas were assessed with the more sensitive but less specific monoclonal anti-pan-TLE, and the MSKCC arrays, which only contained synovial sarcoma cases, were assessed with the less-sensitive M101. TLE staining results for each tumor type are summarized in Table 2.

Of the 35 bone and soft tissue tumors types with at least 5 cases included in this study, synovial sarcomas were the only type displaying a high proportion of positive TLE staining, with 91/94 (97%) cases exhibiting intense and/or diffuse nuclear staining (i.e. 2+ or 3+; Table 2). TLE staining in a core biopsy of a synovial sarcoma is demonstrated in Figure 2. All cases of biphasic synovial sarcoma exhibited staining in both the epithelial and spindle cell components (Figure 1B), with the epithelial component showing equivalent or stronger intensity. Four cases had the histology of poorly-differentiated synovial sarcoma, and all of these were positive for TLE (three cases scored as 3+, one as 2+). For the synovial sarcoma cases with known SSX subtype, all 25 SYT-SSX1 and all 17 SYT-SSX2 were positive for TLE. TLE staining was weak (i.e. 1+) in only 3/94 synovial sarcomas while none of the synovial sarcomas had absent (score = 0) staining. TLE staining in a core biopsy of a synovial sarcoma is demonstrated in Figure 2.

In contrast to synovial sarcoma, TLE staining was low to absent in other spindle cell tumors, including those in the differential diagnosis of synovial sarcoma. Solitary fibrous tumor/hemangiopericytomas (6/20) and schwannomas (6/22) were occasionally positive, whereas fibroxanthoma (1/4), clear cell sarcoma (1/7), carcinosarcoma (1/7), high grade chondrosarcoma (1/8), Ewing sarcoma (1/13 cases), MPNST (4/88), gastrointestinal stromal tumor (1/34) and leiomyosarcoma (1/41) were rarely positive for TLE (Table 2). The remaining tumors in this study, including malignant fibrous histiocytoma (MFH, synonymously termed pleomorphic undifferentiated sarcoma), fibrosarcoma and dermatofibrosarcoma protuberans with fibrosarcomatous change, were negative for TLE staining in all examined cases.

Discussion

Distinguishing synovial sarcoma from other spindle cell tumors can present a diagnostic challenge, particularly in those cases that do not exhibit biphasic histology. In these situations immunohistochemical markers can be valuable in confirming the diagnosis of synovial sarcoma. Many attempts have

been made to identify immunomarkers that have high positive predictive value for synovial sarcoma, but to date there have been no markers identified which are both consistently specific and sensitive for this tumor (12, 15, 27). Keratin and/or EMA immunostains, which are commonly used, are sensitive markers for synovial sarcoma, however their expression is not specific as many tumors, including MPNST, also express epithelial antigens (8, 13, 27).

Gene expression studies in synovial sarcoma have repeatedly shown overexpression of members of the *TLE* family of genes, particularly *TLE1*, in synovial sarcoma. Analysis of this gene expression data has identified *TLE1* as one of the best discriminators for synovial sarcoma when compared to histologically and/or biologically similar sarcomas such as MPNST or Ewing sarcoma. These data, from multiple groups using different expression profiling platforms, suggest that TLE may be a valuable diagnostic marker for synovial sarcoma.

This study demonstrates that immunohistochemical detection of TLE is not only very highly sensitive for synovial sarcoma, but also specific in the context of other mesenchymal neoplasms. Results are consistent with either a monoclonal antibody recognizing pan-TLE or a commercially available polyclonal antiserum that recognizes TLE1 (and cross-reacts to a lesser extent with TLE2, 3 and 4). We defined positive staining for TLE as moderate to high level staining as described in the materials and methods. Even using stringent criteria, the vast majority of synovial sarcomas (97%) were positive. Other tumors commonly mistaken for synovial sarcoma exhibited lower levels of positive staining for pan-TLE, including schwannomas (27%), Ewing sarcomas (8%), MPNST (5%) and MFH (0%). Notably, TLE expression distinguishes synovial sarcoma from MPNST, a particularly problematic entity in the differential diagnosis, with a high degree of specificity. The majority of the remaining tumor types exhibit low to absent levels of TLE immunostaining, suggesting that TLE is useful in differentiating synovial sarcoma from these tumors. Since a high proportion of cells within most of the synovial sarcoma tissue microarray cores exhibited TLE staining (i.e. 2+ or 3+), high sensitivity on small tissue samples, such as core biopsies, would be seen (Figure 2). Using synovial sarcoma tissue microarrays we have now assessed over 35 established and novel immunohistochemical markers of synovial sarcoma (24), none of which perform as well as TLE.

The prominence of *TLE1* and other components of the Wnt/ β -catenin signaling pathway in synovial sarcoma gene expression studies suggest that they may well play an important role in the development of this tumor. The observation that high levels of nuclear-localized β -catenin are a feature of synovial sarcoma (20), and recent experimental data showing that SYT-SSX expression leads to accumulation of β -catenin in a nuclear complex that includes SYT-SSX, provides evidence that downstream components of this pathway that mediate its effects on gene transcription are probably important in the pathogenesis of synovial sarcoma (28). The present study demonstrates protein-level correlation of *TLE1* mRNA overexpression in synovial sarcoma. TLE1 competes with activated β -catenin for TCF/LEF transcription factors in the nucleus; TLE1 binding displaces β -catenin to form transcriptionally repressive TLE1-TCF/LEF complexes (4, 9). A recent study demonstrated that β -catenin and SYT-SSX2 interact to potentiate β -catenin-mediated transcription (28), suggesting that TLE1 overexpression may

represent a compensatory response to excessive β -catenin signaling or serve to limit transcriptional activation to certain genes. TLE is also known to associate with other transcription factors, such as HES, where it fulfills a similar role as an adaptor molecule within multiprotein complexes involved in transcriptional repression (6). In this context, TLE may serve to repress genes involved in differentiation and maintain the relatively undifferentiated histopathologic state seen in synovial sarcoma. Further functional studies are required to delineate the role of TLE proteins in synovial sarcoma pathogenesis.

TLE functions to repress transcription via recruitment of histone deacetylase activity (6). Of interest, recent data have shown strong growth inhibition of synovial sarcoma models that were treated with clinically-applicable histone deacetylase inhibitors (17, 18).

In conclusion, TLE1 expression is a consistent and prominent feature of synovial sarcoma whereas it is low to absent in other tumors in the differential diagnosis. Reproducible immunohistochemical staining of TLE1 with negligible background can be obtained with both monoclonal and commercially available polyclonal anti-TLE1 antibodies. TLE1 is a sensitive and specific immunohistochemical marker for synovial sarcoma, performing better than other known immunohistochemical markers, and can significantly aid in the pathologic diagnosis of this tumor.

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Tables

Study	TLE1 Rank*	Array Type	Ranking Parameter	SS Cases	Comparison Group
Baird <i>et al.</i> Ref #3	5	12600 cDNA spotted	Weighted p-value	16	1 ASPS; 1 CCS; 1 CSa; 5 DFSP; 19 EWS; 7 FSa; 5 GIST; 6 HPCT; 17 LMS; 33 LPS; 38 MFH; 2 MMT; 5 OSa; 6 BS; 3 MPNST; 6 RMS; 10 NOS
Ladanyi Ref #19	1	22283 probe set Affymetrix U133A	Fold change in expression	46	28 EWS; 28 DSRCT; 23 ARMS; 12 ASPS
Nielsen <i>et al.</i> Ref #22	4	42000 cDNA spotted	p-value	13	24 MPNST
Segal <i>et al.</i> Ref #28	2	12626 probe set Affymetrix U95A	p-value	5	6 CCS; 5 DDLS; 5 GIST; 8 FSa; 6 LMS; 11 MFH; 3 PLS; 4 RCLS

ARMS: Alveolar rhabdomyosarcoma; ASPS: Alveolar soft parts sarcoma; BS Benign schwannoma; CCS: Clear cell sarcoma; CSa: Chondrosarcoma; DFSP: Dermatofibrosarcoma protuberans; DDLS: Dedifferentiated liposarcoma; DSRCT Desmoplastic small round cell tumor; EWS: Ewing sarcoma; FSa: Fibrosarcoma; GIST: Gastrointestinal stromal tumor; HPCT: Hemangiopericytoma; LMS: Leiomyosarcoma; LPS: Liposarcoma; MFH: Malignant fibrous histiocytoma; MMT: Malignant Mixed Mullerian tumor; MPNST: Malignant peripheral nerve sheath tumor; NOS: Unclassified sarcoma; OSa: Osteosarcoma; PLS: Pleiomorphic liposarcoma; RCLS: Round-cell liposarcoma; RMS: Rhabdomyosarcoma; SS: Synovial sarcoma

* rank of TLE1 within total gene list when sorted by ability to positively discriminate synovial sarcoma

Table 1 Gene microarray expression profiling studies identify *TLE* as a good discriminator for synovial sarcoma from other sarcomas.

Tumor Type	n	3+	2+	1+	0	Total Positive	% Positive
Synovial Sarcoma	94	70	21	3	0	91	97
Hemangiopericytoma	5	1	1	1	2	2	40
Schwannoma, regular	16	0	5	7	4	5	31
Solitary Fibrous Tumor	15	0	4	3	8	4	27
Fibroanthoma	4	0	1	0	3	1	25
Schwannoma, cellular	6	0	1	4	1	1	17
Carcinosarcoma	7	0	1	2	4	1	14
Clear Cell Sarcoma	7	1	0	1	5	1	14
Chondrosarcoma, high grade	8	0	1	0	7	1	13
Ewing Sarcoma	13	1	0	1	11	1	8
GIST	35	0	2	7	26	2	6
MPNST	88	1	3	12	72	4	5
Leiomyosarcoma	41	1	0	4	36	1	2
Angiosarcoma	13	0	0	0	13	0	0
Breast Carcinoma	2	0	0	0	2	0	0
Chondroblastoma	4	0	0	1	3	0	0
Chondromyxoid Fibroma	2	0	0	0	2	0	0
Chondrosarcoma, myxoid	7	0	0	2	5	0	0
Chondrosarcoma, low grade	23	0	0	0	23	0	0
Chondrosarcoma, mesenchymal	2	0	0	0	2	0	0
DFSP*	17	0	0	1	16	0	0
DSRCT	3	0	0	0	3	0	0
Enchondroma	4	0	0	0	4	0	0
Endometrial Stromal Sarcoma	11	0	0	1	10	0	0
Epithelioid Sarcoma	2	0	0	0	2	0	0
Fibromatosis	17	0	0	0	17	0	0
Fibrosarcoma	3	0	0	0	3	0	0
Giant Cell Tumor, tenosynovial	9	0	0	0	9	0	0
Glomous Tumor	7	0	0	1	6	0	0
Granular Cell Tumor	8	0	0	0	8	0	0
Vascular Tumor **	9	0	0	0	9	0	0
Inflammatory Myofibroblastic Tumor	3	0	0	0	3	0	0
Kaposi Sarcoma	2	0	0	0	2	0	0
Leiomyoma	8	0	0	0	8	0	0
Lipoblastomatosis	2	0	0	0	2	0	0
Liposarcoma, dedifferentiated	14	0	0	1	13	0	0
Liposarcoma, myxoid	4	0	0	1	3	0	0
Liposarcoma, pleomorphic	2	0	0	0	2	0	0
Liposarcoma, well differentiated	11	0	0	1	10	0	0
MFH	56	0	0	5	51	0	0
Melanoma	11	0	0	4	7	0	0
Myxofibrosarcoma, low grade	5	0	0	0	5	0	0
Myxoma	8	0	0	0	8	0	0
Myxosarcoma	4	0	0	1	3	0	0
Neurofibroma, diffuse	12	0	0	4	8	0	0
Neurofibroma, localized	8	0	0	1	7	0	0
Neurofibroma, plexiform	24	0	0	5	19	0	0
Osteosarcoma	16	0	0	1	15	0	0
Perineurioma	4	0	0	0	4	0	0
Rhabdomyosarcoma	13	0	0	2	11	0	0
Sarcoma, NOS	7	0	0	1	6	0	0

DFSP: Dermatofibrosarcoma Protuberans, DSRCT: Desmoplastic Small Round Cell Tumor, GIST: Gastrointestinal Stromal Tumor, MFH: Malignant Fibrous Histiocytoma, MPNST: Malignant Peripheral Nerve Sheath Tumor, NOS: Not Otherwise Specified

* Eight of the DFSP cases contained fibrosarcomatous change

** Includes 2 cases of epithelioid hemangioendothelioma, 4 capillary hemangiomas and 3 intramuscular hemangiomas.

Table 2 Summary of TLE immunohistochemistry results.

Figure Legends

Figure 1 TLE immunostaining in representative 0.6 mm cores using pan-TLE antibody. A) 3+ staining in monophasic synovial sarcoma. B) higher power view of 2+ staining in a biphasic synovial sarcoma. C) 1+ staining in MPNST. D) Negative (score = 0) staining in MPNST.

Figure 2 Monoclonal anti-pan-TLE immunostaining of a core needle biopsy, taken from a 6 cm wrist mass in a 49 year-old male. H&E showed a nonpleomorphic spindle cell sarcoma, which was negative for pankeratin, CK7 and EMA by routine diagnostic immunohistochemistry protocols. Subsequent diagnostic FISH assay was positive for a split at the SYT locus, confirming synovial sarcoma 9 days after the TLE immunostaining result. Main image 1.25x, inset 40x. Full biopsy slide available for viewing at <https://www.qpecimage.ubc.ca/tma/web/viewer.php>

Figure 1
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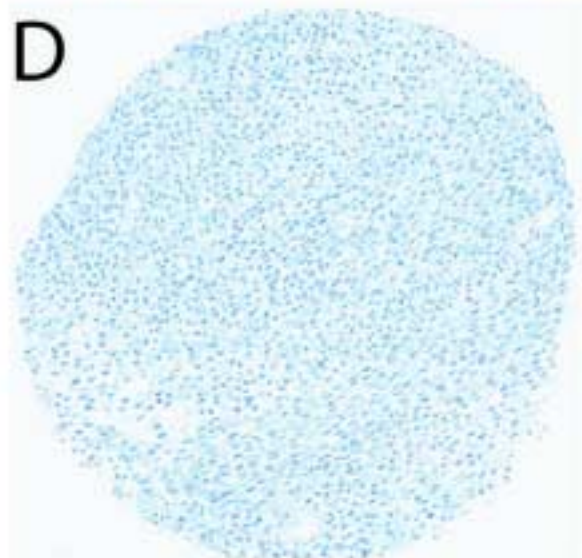
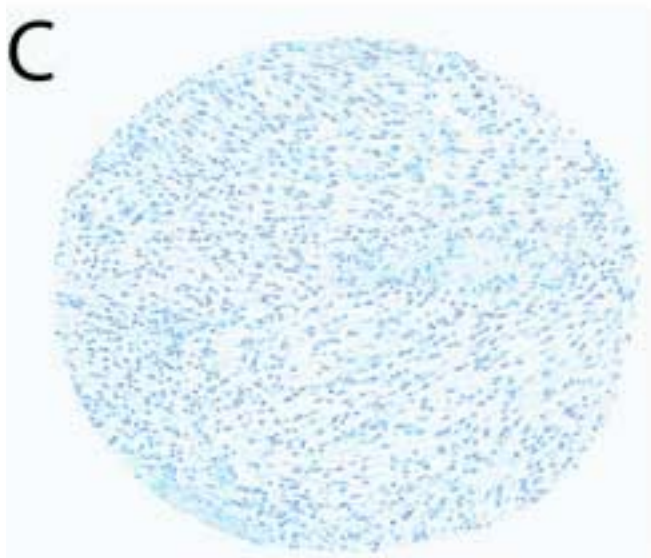
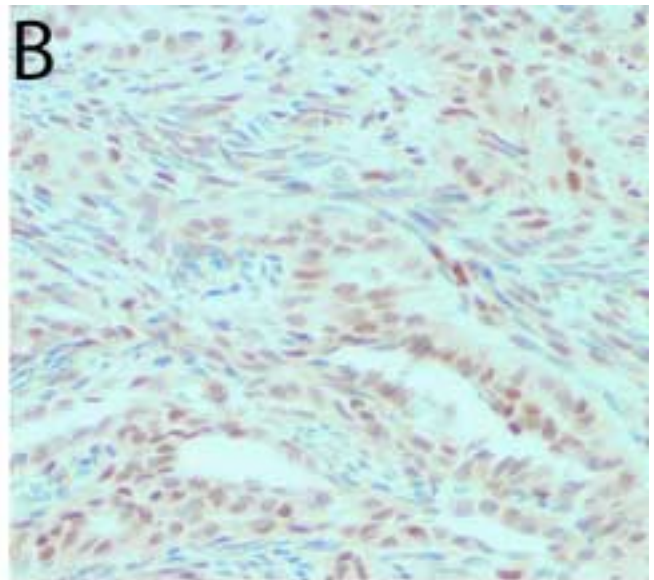
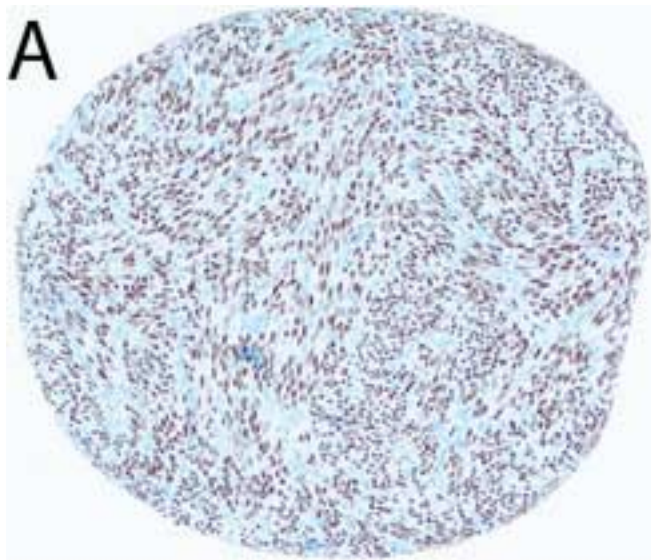


Figure 2

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