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Expression profiling of formalin-fixed paraffin embedded primary human uveal melanomas using DASL matrices

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Abstract

Purpose Fresh biopsied ocular tumor tissues are difficult to obtain for the purpose of performing microarray experiments on extracted nucleic acids. Present technology allows for extraction of total RNA from formalin-fixed paraffin embedded (FFPE) tissue analyzed by the cDNA mediated Annealing Sectioning and Ligation (DASL) method. We aimed to correlate gene transcript differences between two uveal melanoma (UM) clinical–histopathological parameters (metastasis, cell type).

Methods A total of 43 FFPE UM were used. The expression of RPL13a, a ribosomal protein gene, for each sample was used to evaluate the quality of RNA extracted from

FFPE tissue. Gene expression values generated from the array were analyzed using the GeneSpring GX software (Agilent). Immunohistochemistry was used in order to validate transcriptional findings at the protein level.

Results A total of 106 genes were identified with ($P < 0.05$, Welch ANOVA test) a difference in transcript abundance for the metastasis clinical parameter. Furthermore, we identified 64 genes with a statistically significant ($P < 0.05$) difference in transcript abundance between the spindle and epithelioid cell types. Each individual sample for both groups (metastasis, cell type) exhibited distinct transcriptional profiles that were separated on a PCA. Positive nuclear immunostaining for LIG4-metastasis, ErbB3-cell type was found to be associated with better patient prognosis and outcome.

Conclusions To the best of our knowledge, this is the first time that a successful retrospective analysis has been done with UM FFPE RNA. This data may lead to future customized therapeutic targets, which may improve the now unchanged mortality rate of this particular malignancy.

Keywords Uveal melanoma · FFPE · DASL · Expression profiling · Histopathology · Clinical parameters

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Introduction

Many of the underlying genetic causes of uveal melanoma (UM) metastatic disease still remain to be elucidated. This particular cancer is the most common primary malignant intraocular tumor in adults, comprising approximately 5% of all diagnosed melanoma cases (Diener-West et al. 1992; Singh 2007). Despite its rarity, roughly 50% of patients diagnosed with UM will develop metastatic disease (Diener-West et al. 1992). In addition, due to the lack of

lymphatics in the eye, this tumor disseminates almost exclusively via the hematogenous route. More than 60.5% of patients with metastatic disease will end up developing it in the liver (Rietschel et al. 2005).

Advances in genetic and in vitro analysis in recent years have revealed many novel insights into the genetic causes of UM metastasis. High-throughput technologies, including cDNA microarrays and aCGH array analysis, have been the focus of many recent papers that have identified various “molecular-genetic signatures” for this particular neoplasm (van der Velden et al. 2003; Zuidervaart et al. 2003; Onken et al. 2004; Marshall et al. 2007; Meir et al. 2007; Singh et al. 2007). While these papers have presented very consistent data that identify significant chromosomal aberrations in chromosome 3, 6 and 8 (Aalto et al. 2001; White et al. 2006; Onken et al. 2007; Mensink et al. 2008; van Gils et al. 2008), the tissue used for these transcriptional studies were mostly fresh frozen and are more difficult to acquire due to the fact that enucleation is no longer the primary treatment for UM.

This study attempts to “unlock” the potential of using RNA extracted from the very plentiful reservoir of UM’s that is formalin-fixed and paraffin embedded. This particular method will also allow the investigator to correlate the transcriptional profiling data with well documented clinical–histopathological data, and patient follow up which is much more difficult to obtain when using fresh tissue samples. The ability to extract RNA from archival sources allows us to have a very plentiful reserve of tissue that can date back as far as 12 years. This method also allows for large scale analysis of many different types of UM, which in turn increases a studies reproducibility and statistical power.

It is well known that DNA and RNA integrity are greatly reduced once tissue is treated with formalin. The formalin fixation process creates extensive cross-linking between nucleic acids and cellular proteins (Li et al. 2007). These chemical modifications are irreversible and damage nucleic acid integrity (Li et al. 2007). RNA fragmentation and degradation can also occur during other steps of the tissue archiving processes such as the delay between tissue acquisition and time of fixation. A factor which frequently varies between pathology laboratories (von Ahlfen et al. 2007). Autolysis, due to improper handling and temperature sensitivity will also extensively affect the quality of RNA within the tissue prior to formalin fixation.

Recent advances in microarray technology now allow for high-throughput analysis of partially degraded RNAs from FFPE tissues via the cDNA mediated Annealing Sectioning and Ligation (DASL) PCR method. Typically, RNAs extracted from FFPE tissues can yield qPCR amplicons ranging from 50 to 700 nucleotides in length (Bibikova et al. 2007; von Ahlfen et al. 2007). By targeting anywhere

between 3 and 10 sites per gene with DASL probes, this allows for a greater probability of detecting sequences that have been partially degraded through the FFPE process.

Previous studies in other malignancies have used FFPE material for RNA extraction and gene expression profiling (Bibikova et al. 2004, 2007). In both studies, FFPE blocks that were up to 11 years of age at the time the study were used. The most recent study correlated transcriptional variances with the well characterized Gleason score for prostate cancer (Bibikova et al. 2007). The genes involved correlate significantly ($P = 0.007$) with disease relapse when tested with a gene expression score. These results further proved the reliability and reproducibility of the platform and RNA extraction methods used.

In this study, we aim to investigate the feasibility of extracting partially degraded RNAs from 43 FFPE primary human UM. We also aim to correlate gene expression profiles obtained from DASL analysis to specific oncogene targets with well documented clinical and histopathological parameters in UM. This will in turn reveal a greater understanding of the molecular components that may distinguish more benign UM from the more aggressive-malignant counterpart.

Materials and methods

Specimens

UM specimens were obtained by enucleation between 1994 and 2005 were collected from the archives of Instituto Universitario de Oftalmobiología Aplicada (IOBA), University of Valladolid, Eye Pathology Laboratory. Each specimen was FFPE, and contained sufficient material to produce 10 μM sections. Sections were placed in an Eppendorf tube for later extraction. Tumors presenting extensive necrosis that precluded an appropriate evaluation of histopathological features were excluded. Histopathological analysis of the specimens with regards to prognostic factors such as cell type (modified Callender’s classification), scleral invasion, and tumor location were determined. Clinical follow up including date of diagnosis, laterality of the tumor, date and motive of patient death are also known.

RNA extraction and validation

A total of 43 FFPE primary human UM were used to extract RNA. Each block was sectioned to a thickness of 10 μM and each section was placed in a 1.5 ml Eppendorf tube. The High Pure RNA Paraffin Kit (Roche) was used to extract total RNA from each of the 10 μM tissue sections. This procedure involves deparaffinization, cell lysis, separation, DNase treatment, purification, and elution. All

samples were stored at -80°C until used for further analysis. The quantity and quality of the total RNA extracted from each specimen was evaluated using a spectrophotometry, quantitative real-time PCR (qPCR), and capillary electrophoresis. The concentration of each sample ($\text{ng}/\mu\text{l}$) along with the purity ratio (O.D: 260/280) was evaluated in a NanoDrop Spectrophotometer (ND-3300). The expression of RPL13a, a ribosomal protein gene, was also assessed based on an optimized protocol developed to specifically evaluate the quality of RNA extracted from FFPE tissue (Illumina Systems). The forward (F) and reverse (R) sequence of the RPL13a primers (F: 5' GTACGCTGTG AAGGCAATCAA 3' R: 5' GTTGGTGTTCATCCGCTTG 3') were used in all qPCR experiments (IDT). Illumina single use cDNA synthesis kit was used to reverse transcribe all samples for qPCR. High quality RNA, when normalized to $40 \text{ ng}/\mu\text{l}$, was considered to be of acceptable quality when amplification occurred between an average cross-threshold (C_t) of 22–28 PCR cycles. Finally, the Eukaryote Total RNA Pico Series II Bioanalyzer Chip (Agilent) was also used to verify RNA quality in 11 randomly chosen samples.

DASL cancer panel array

All RNA samples validated were then processed and analyzed using the DASL PCR method (Illumina) as previously described (Bibikova et al. 2004; Fan et al. 2004; Steemers and Gunderson 2005). The cancer panel platform was used, which includes a pool of selected probe groups that target 502 genes collected from ten publicly available cancer gene lists. Each gene in the Cancer Panel is targeted in three locations with three probe groups per gene (Fan et al. 2004; Steemers and Gunderson 2005). This array was performed on all 43 samples by previously described methods (Steemers and Gunderson 2005). The GeneSpring GX software version 7.3 (Agilent Technologies) was used to analyze the results obtained. Every sample used in this experiment was performed in duplicate.

Immunohistochemistry

Immunohistochemistry was performed using the Ventana BenchMark fully automated machine. The fully automated processing of bar code labeled slides included baking of the slides, solvent-free deparaffinization, and CC1 (Tris/EDTA buffer pH 8.0) antigen retrieval. Slides were incubated with a polyclonal mouse anti-human LIG4 antibody (dilution 1:50; Abcam Inc., Cambridge, MA, USA;) for 30 min at 37°C , followed by application of biotinylated secondary antibody (8 min at 37°C) and an avidin/streptavidin enzyme conjugate complex (8 min at 37°C). Finally, the antibody was detected using

the Fast Red chromogenic substrate and counterstained with hematoxylin. As positive controls, sections of human adrenal glands and liver were used for the LIG4 antibody. For negative controls the primary antibody was omitted. Sections were analyzed for LIG4 expression in tumor samples and surrounding ocular tissues. An additional validation set of 15 FFPE primary UM were used for this analysis. Slides were incubated with a monoclonal [RTJ2] mouse anti-human ErbB3 antibody (dilution 1:100; Abcam Inc., Cambridge, MA, USA;) for 30 min at 37°C , followed by application of biotinylated secondary antibody (8 min at 37°C) and an avidin/streptavidin enzyme conjugate complex (8 min at 37°C). Finally, the antibody was detected using the Fast Red chromogenic substrate and counterstained with hematoxylin. As positive controls, sections of human breast tumor and prostate cancer were used for the ErbB3 antibody. For negative controls the primary antibody was omitted. Sections were analyzed for LIG4 and ErbB3 expression in tumor samples and surrounding ocular tissues. A total of 15 FFPE primary UM were used for this analysis for each antibody in question. All samples were from an independent sample set and were in no way related to samples used for the DASL analysis. This was done in order to insure that results could be successfully reproduced in an independent data set. Samples were also independently graded for percentage of sample nuclear and cytoplasmic positivity for LIG4 and ErbB3 by two different pathologists.

Statistical analysis

A common detection score test was used to assess the average number of targets detected in the DASL cancer panel (>0.99). A Welch ANOVA test ($P < 0.05$) was used to assess differences in transcript abundance between patients that died of metastasis versus those that did not die from metastasis and as well as between tumors that were mostly composed of spindle versus epithelioid cell types. Alternatively, interesting gene markers were also identified using the GeneSpring Class Predictor tool (K-nearest neighbors, gene selection method: Fisher's exact test, number of genes: 10 for cell type, 12 for death, number of neighbors: 8, decision cutoff for P value ratio: 0.2). A Kaplan–Meier survival analysis test ($P < 0.05$, MedCalc® V 10.3.0.0) was used to assess the significance of LIG4 immunohistochemical staining patterns and to validate the DASL cancer panel data. Results from the ErbB3 staining were interpreted using a Correlation analysis (MedCalc® V 10.3.0.0). A correlation was drawn by comparing ErbB3 staining intensity and UM cell type (spindle, epithelioid). A result was considered significant when a P value of <0.05 was obtained.

Results

RNA validation

All 43 samples used for RNA extraction yielded RNA concentrations ranging from 13.0 to 198.8 ng/μl. Samples must have an optimal concentration of 40 ng/μl in order to pass DASL assay criteria. Of the 43 samples assayed, 29 (67%) met the minimum 40 ng/μl concentration criteria. Using qPCR, RPL13a could be amplified in each of the 43 RNA samples indicating the presence of intact usable RNAs that were not extensively degraded. Ct values for the specimens ranged from 25 to 35 qPCR cycles (Fig. 1b). A standard melting curve analysis demonstrated the presence of a very uniform amplification product for all samples tested (Fig. 1c). Bioanalyzer results showed that RNA was present in all of the 11 samples tested (Fig. 1d). Prominent 18S and 28S ribosomal peaks were present on 4 out of the 11 samples indicating relatively intact, quality RNA. In addition, a negative correlation was observed when comparing the age of the block to the concentration of RNA extracted from that block ($r = -0.4137$, $P = 0.006$) (Fig. 1e). Furthermore, all concentrations for every sample was plotted out in a box and whisker diagram in order to visually represent the population of samples that passed to those that failed the 40 ng/μl concentration cutoff (Fig. 1f).

DASL cancer panel

All samples were tested for mean fluorescent intensity of signal strength. Despite the fact that only 29 of 43 samples passed initial RNA quality tests, all 43 were tested for signal strength and 41 were subsequently deemed suitable for analysis. This increased the total pass rate of the samples from 67 to 95%. We disregarded the remaining two samples due to very poor reproducibility of signal strength between the two replicates. A total average of 366 genes out of 502 were detected for all 43 UM samples analyzed (>0.99 using a common detection score calculation). All known clinical and histopathological parameters were imputed into the GeneSpring GX software and we used two different strategies to associate gene clusters to known parameters, most notably metastasis free survival, and cell type. ANOVA testing identified 106 genes with a significant change in transcript abundance ($P < 0.05$, Welch *T* test) between tumors from patients that died of metastasis to those that did not. The top 11 gene candidates for the metastasis parameter ($P < 0.03$) are displayed in Table 1. Similarly, 64 genes ($P < 0.05$, Welch *T* test) significantly differed in transcript abundance when the spindle and epithelioid cell type parameters were compared. Tumors with a mixed cell population were not included in this analysis. The top ten gene candidates for the cell type parameter

($P < 0.002$) are listed in Table 2. We also tested various gene list sizes ($n = 5$ –20) in the GeneSpring Class Prediction tool. As a predictor of metastasis-dependant death, 12 genes listed in Table 3, were shown to correctly predict this parameter in 27/37 tumors (73%). Table 4 also includes a list of 10 genes that successfully predicted the classification of 27/31 (87.1%) non-mixed tumors. The ability of these two lists of classifier genes to separate to various UM tumors is illustrated in Fig. 2a, b as a principal components analysis (PCA).

Immunohistochemistry

Based on the analysis of gene expression data, the LIG4 and ErbB3 genes were consistently ranked as highly distinctive markers, irrespective of the statistical method that was used. We thus decided to validate their protein expression in an independent set of UM tumors. Very strong nuclear staining for LIG4 was observed in all spindle cell UM for 15 independent samples stained. The nuclear staining sharply decreased when observing epithelioid cell UM samples. This data is very consistent with the mRNA expression pattern observed in the DASL Cancer panel analysis of DNA Ligase IV (LIG4). Mixed cell type UM samples also display the same relationship with strong nuclear staining observed in the spindle cell type population while epithelioid cell populations are almost completely devoid of any visible nuclear stain for LIG4. Moderate to weak cytoplasmic staining of LIG4 was seen uniformly in all UM cell types that were stained. The data generated from immunohistochemical analysis validates the transcriptional differences seen in the DASL assay preformed on all 43 FFPE UM samples. Furthermore, the expression of LIG4 was positive in all 15 (100%) specimens examined. Staining pattern was diffuse in 13 (86.65%) and focal in 2 (13.3%) cases. Positive nuclear immunostaining for LIG4 was associated with a better distant metastasis free survival rate (Kaplan–Meier test $P = 0.0215$) (Fig. 3). Very strong nuclear staining for ErbB3 was observed in all spindle cell UM for 15 independent samples stained. The nuclear staining sharply decreased in epithelioid cell UM samples. This data is very consistent with the mRNA expression pattern observed in the DASL Cancer panel analysis of ErbB3 (HER3). Mixed cell type UM samples also display the same relationship with strong nuclear staining observed in the spindle cell type population while epithelioid cell populations are almost completely devoid of any visible nuclear stain for LIG4. Moderate to weak cytoplasmic staining of ErbB3 was seen uniformly in all UM cell types that were stained. The data generated from immunohistochemical analysis validates the transcriptional differences seen in the DASL assay preformed on all 43 FFPE UM

Fig. 1 Analysis of FFPE RNA extracted from 43 Primary Human UM. **a** A qPCR graph displaying strong reproducibility between samples replicates when amplifying RPL13a gene (amplified between 22 and 28 cycles). **b** A graph displaying the range of C(t) values for all 43 samples analyzed. The two dots at around C(t) 18 are control cell line (92.1) RNA. **c** Melting curve analysis of RPL13a 90 base pair

amplicon for all 43 FFPE UM. **d** Bioanalyzer results for all 11 samples tested. **e** A negative correlation displaying diminishing RNA concentration yield when the age of the block increases. **f** Box and whisker plot representing the average RNA concentrations (ng/μl) of all 43 samples (40 ng/μl cutoff indicated)

Table 1 Top 11 metastasis candidates (Welch ANOVA test)

	Gene name	<i>P</i> value
1	Ligase IV; DNA; ATP-dependent (LIG4)	0.000603
2	Methyl-CpG binding domain protein 2 (MBD2); transcript variant testis-specific	0.00118
3	ELK3; ETS-domain protein (SRF accessory protein 2) (ELK3)	0.00673
4	Insulin-like growth factor 2 receptor (IGF2R)	0.0112
5	MLL septin-like fusion (MSF)	0.0149
6	Nijmegen breakage syndrome 1 (nibrin) (NBS1)	0.0152
7	Topoisomerase (DNA) II alpha 170 kDa (TOP2A)	0.0211
8	Fibroblast growth factor 9 (glia-activating factor) (FGF9)	0.0287
9	v-myc myelocytomatosis viral oncogene homolog (avian) (MYC)	0.0325
10	Cyclin-dependent kinase inhibitor 2A (melanoma; p16; inhibits CDK4) (CDKN2A); transcript variant 2	0.0326
11	ets variant gene 6 (TEL oncogene) (ETV6)	0.0394

Table 2 Top 10 cell type candidates (Welch ANOVA test)

	Gene name	<i>P</i> value
1	v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian) (ERBB3)	2.17E-06
2	Insulin-like growth factor binding protein 2 (36kD) (IGFBP2)	0.0000859
3	Insulin-like growth factor 2 receptor (IGF2R)	0.000205
4	ELK3; ETS-domain protein (SRF accessory protein 2) (ELK3)	0.000731
5	Fibroblast growth factor 9 (glia-activating factor) (FGF9)	0.000923
6	Nijmegen breakage syndrome 1 (nibrin) (NBS1)	0.00118
7	Breakpoint cluster region (BCR); transcript variant 1	0.00168
8	Friend leukemia virus integration 1 (FLI1)	0.00215
9	Frizzled homolog 7 (Drosophila) (FZD7)	0.00222
10	Werner syndrome (WRN)	0.00232

Table 3 Class prediction for metastasis (K-nearest neighbors algorithm)

	Gene name	Predictive strength
1	Ligase IV; DNA; ATP-dependent (LIG4)	11.01
2	Kinase insert domain receptor (a type III receptor tyrosine kinase) (KDR)	9.473
3	Epidermal growth factor receptor pathway substrate 15 (EPS15)	7.724
4	MLL septin-like fusion (MSF)	7.724
5	Transforming growth factor; beta 1 (Camurati-Engelmann disease) (TGFB1)	7.1
6	Insulin-like growth factor 1 receptor (IGF1R)	7.1
7	Tumor necrosis factor receptor superfamily; member 1A (TNFRSF1A)	7.1
8	Sema domain; immunoglobulin domain (Ig); short basic domain; secreted; (semaphorin) 3F (SEMA3F)	6.311
9	PTK2 protein tyrosine kinase 2 (PTK2); transcript variant 1	6.209
10	Platelet-derived growth factor receptor; beta polypeptide (PDGFRB)	6.209
11	Hepatoma-derived growth factor (high-mobility group protein 1-like) (HDGF)	6.209
12	Cas-Br-M (murine) ecotropic retroviral transforming sequence b (CBLB)	6.066

samples. The percentage of nuclear staining is also far greater (80–90%) in patients with spindle cell type UM when compared to patients who had epithelioid cell type UM (0–5%) (Fig. 4).

Discussion

This study is the first to show that total RNA extracted from FFPE UM tissues can be used for high-throughput

Table 4 Class prediction for cell type (K-nearest neighbors algorithm)

	Gene name	Predictive strength
1	v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian) (ERBB3)	11.11
2	Baculoviral IAP repeat-containing 3 (BIRC3); transcript variant 2	8.983
3	Frizzled homolog 7 (Drosophila) (FZD7)	8.491
4	Hemopoietic cell kinase (HCK)	7.374
5	v-yes-1 Yamaguchi sarcoma viral oncogene homolog 1 (YES1)	7.22
6	Ligase IV; DNA; ATP-dependent (LIG4)	7.061
7	Insulin-like growth factor binding protein 2 (36kD) (IGFBP2)	7.021
8	Insulin-like growth factor 2 receptor (IGF2R)	6.605
9	Neogenin homolog 1 (chicken) (NEO1)	6.605
10	MRE11 meiotic recombination 11 homolog A (<i>S. cerevisiae</i>) (MRE11A); transcript variant 1	6.223

Fig. 2 Principal components analysis of 36 FFPE UM. **a** PCA displaying spatial separation (transcriptional clustering) of patients that have died from UM metastatic disease to those that have not. **b** PCA displaying spatial separation of patients that are predominantly spindle to those that are epithelioid and those of mixed cell types

Fig. 3 Immunohistochemical validation of DNA ligase IV. **a** Nuclear positivity for LIG4 in a spindle type UM ($\times 200$). **b** Epithelioid section displaying negative staining for LIG4 ($\times 200$). **c** DASL transcriptional data displaying a decrease in LIG4 mRNA expression from spindle > mixed > epithelioid. **d** Kaplan–Meier survival analysis displaying better distant free metastasis for patients that stained positive for LIG4

Fig. 4 Immunohistochemical validation of ErbB3. **a** Nuclear positivity for ErbB3 in a spindle type UM ($\times 200$). **b** Epithelioid section displaying decreased nuclear staining for ErbB3 ($\times 200$). **c** DASL transcriptional data displaying a decrease in ErbB3 mRNA expression from spindle > mixed > epithelioid. **d** Histogram depicting percentage of ErbB3 nuclear staining comparing patients that have spindle cell type compared to those that had epithelioid cell type. Correlation analysis revealed a positive correlation ($n = 15$, $r = 0.9121$, $P < 0.001$) between nuclear positivity of ErbB3 and spindle cell type UM

microarray analysis in order to determine expression level relationships between different UM tumor types. This is particularly useful in determining specific factors that are up-regulated in more aggressive UM primary tumor types. Such studies will intern also identify novel genetic targets (gene clusters/groups) that correlate well with known and established clinical and histopathological parameters. Our study has also attempted to add to the current DNA damage/repair mechanism theory that has been postulated for UM tumor progression. Previous reports have identified NBS1 as an important prognostic marker in UM progression and pathogenesis (Ehlers and Harbour 2005). This particular protein (NBS1, Nijmegen breakage syndrome 1) is involved directly in DNA damage repair, and is highly up-regulated in more aggressive “type-2” melanomas (Onken et al. 2004). The authors postulate that over-expression of NBS-1 in more aggressive UM leads to greater genomic stability of that particular cell phenotype. We have also shown that the DNA Ligase IV protein, directly involved in DNA damage repair, to be down-regulated in more aggressive UM tumor types. As previously mentioned, nuclear staining for LIG4 sharply decreased in epithelioid UM cells when compared to spindle type UM cells (Fig. 3). Interestingly, the transcriptional abundance of TOP2A and NBS also increases from spindle < mixed < epithelioid significantly ($P < 0.05$). TOP2A is a well characterized protein that alters the transcriptive activity of DNA by cleaving and re-joining strands to render them more transcriptively active (Lang et al. 1998). In addition, it is of particular

interest in cancer research as there are many therapeutic agents available to inhibit this particular enzyme (Jarvinen and Liu 2003). A recent study has shown that an increase in TOP2A expression positivity correlated with chemotherapeutic resistance ($P = 0.029$) and a marked decrease in patient survival ($P < 0.0001$) (Wong et al. 2008). In concert with NBS1, TOP2A activity may promote genetic stability, and resistance to radiotherapy of the altered “invasive phenotype” of epithelioid cells. It has been a recurrent modality in this array, that DNA damage proteins are highly detectable at all stages of this disease.

Interestingly ErbB3/HER3 was also a highly expressed in the spindle cell type tumors for all samples analyzed. ErbB3 has been previously reported to act as a tight regulator of cell growth and cell differentiation (Liu et al. 2000). ErbB3 is also known to be a part of the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases (Jackson-Fisher et al. 2008). As previously discovered, once the ErbB3 oncogene is activated it is strongly associated with pro-survival signaling via the (PI3K)/Akt Pathway (Jackson-Fisher et al. 2008). Previous work in our laboratory has demonstrated that immunohistochemical expression of phospho-Akt was significantly associated with worse prognosis and metastatic disease (Kaplan-Meier analysis, $P = 0.02$) (Saraiva et al. 2005). It is important to note that ErbB3 was significantly up-regulated in the spindle and mixed cell type when compared to predominantly epithelioid tumor types. It has previously been reported in tumors undergoing epithelioid to mesenchymal

transitional tumors (EMT), particularly cutaneous melanoma, that ErbB3 up-regulation in expression is strongly associated with worse prognosis (Ekberg et al. 2005; Wei et al. 2007; Reschke et al. 2008). UM being a mesenchymal to epithelioid transitional tumor (MET), displays the reverse correlation in staining pattern which was an interesting observation.

The top selected targets LIG4 and ErbB3 were immunohistochemically validated in 15 independent UM samples. These findings correlated well with RNA transcriptional levels leading us to believe that the DASL method is accurate. This method has not only accurately produced reproducible data between replicates, but it has also correlated many novel molecular oncogenic targets with well established clinical and histopathological parameters. This study attempts to give novel insights into UM molecular pathogenesis while identifying key oncogenic mediators with well established parameters that can better predict patient mortality. This may, in the future, give investigators a more focused approach when trying to validate or characterize new molecular prognostic indicators as well as establishing novel molecular therapies. In addition, this technology allows us to access a very large tissue reservoir in a high-throughput fashion, which was in the past only possible with scarcer fresh tissue samples.

This study aims to correlate novel molecular targets with well established parameters associated with worse patient prognosis. Determining a link between these molecular targets and indicators of worse prognosis may lead to promising future therapies. This may hopefully improve the now unchanged mortality rate of this rare aggressive malignancy.

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