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Quantitative Proteomic Analysis of Uveal Melanoma

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Abstract

Purpose. Proteomic analyses of class I low-risk and class 2 high-risk uveal melanoma intraocular tumors were pursued for insights into mechanisms of metastasis from class 1 low-risk to class 2 high-risk and for the identification of biomarkers to detect those at high risk of metastasis.

Methods. Tumor specimens were collected from the eyes of uveal melanoma patients undergoing enucleation and classified based on fluorescence *in situ* hybridization (FISH) analysis. Bruch's membrane choroid tissues were isolated from normal human control postmortem eyes. Proteins were extracted from the tissues in SDS, digested with trypsin, and peptides were labeled with iTRAQ tags. A pooled control sample was prepared from equal amounts of 9 control digests labeled with the same iTRAQ tag. Fifteen tumor digests were combined individually with the pooled control sample (100 µg each) and fractionated by strong cation exchange chromatography. Proteins were identified by LC MS/MS using a Mascot search engine and the Swiss-Protein sequence database. Relative protein quantification from iTRAQ labeling utilized code written in the statistical program R. Bioinformatic pathway analysis was performed with Ingenuity Pathways Analysis 5.0.

Results. Relative protein quantitation has been obtained from 7 class I low-risk and 8 class 2 high-risk uveal melanoma tumors. The LC MS/MS analyses yielded quantification of 1724 proteins by two or more unique peptides per protein, including1473 proteins from class 1 low-risk and 1464 proteins from class 2 high-risk tissues. Ninety-seven proteins from class 1 low-risk and one hundred and eight from class 2 high-risk tumors were quantified in ≥ 3 donor tissues with average ratios ≥ 1 SD above the mean, including macrophage migration inhibitory factor. One hundred and twenty-nine proteins from class 1 low-risk and one hundred and forty-four from class 2 high-risk were quantified in ≥ 3 donor tissues with ratios ≥ 1 SD below the mean, TIMP3 was also of very low abundance in both class 1 low-risk and class 2 high-risk uveal melanoma. Among those differentially expressed proteins in uveal melanoma, fifty-one are unique to class 1 low-risk and seventy-six are unique to class 2 high-risk.

Conclusions. Elevated levels of macrophage migration inhibitory factor in uveal melanoma is consistent with findings from other studies as is decreased amounts of TIMP3 (2007 Br J Ophthmol 91,1385). Proteomic comparisons of class 1 low-risk and class 2 high-risk uveal melanoma suggest possible biomarkers of metastasis.

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Introduction

Uveal melanoma is the most common intraocular cancer in adults. Uveal melanoma is a cancer of the eye involving the iris, ciliary body, or choroid. It is categorized into class 1 with low metastatic risk and class 2 with high metastatic risk. The survival rate of patients with uveal melanoma is poor and has changed little during the past few decades. We compared the proteomes of uveal melanoma Bruch's membrane/choroid tissues with those of control Bruch's membrane/choroid tissues for insights into the molecular mechanism of metastasis and to discover biomarkers to detect those at high risk of metastasis.

Methods

Human Tissues and Sample Preparation. Tumor specimens were collected from the eyes of uveal melanoma patients (n = 15) undergoing enucleation. Based on fluorescence *in situ* hybridization (FISH) analysis, tumor tissues with monosomy 3 and/or 8q amplification were categorized as class 2 high-risk (metastasized) and those without as class 1 low-risk. FISH analysis indicated 7 tumor specimens were from class 1 low-risk and 8 tumor specimens were from class 2 high-risk. Bruch's membrane choroid tissues were isolated from normal human postmortem donor eyes. Protein was extracted in 2% SDS and quantified by the BCA assay. The control reference sample was prepared by combining the same amount of Bruch's membrane/choroid protein from 9 control donors. Protein was precipitated with 4 volumes of acetone to lower the SDS concentration, redissolved in 500 mM TEAB containing 0.1 % SDS, reduced with TCEP, cysteines alkylated with methyl methanethiosulfonate and the alkylated protein (100 µg) was digested with trypsin.

SCX Fractionation and iTRAQ Labeling. Tryptic peptides from the pooled reference sample and the individual tumor samples were labeled with different iTRAQ tags and approximately equal amounts of each were mixed together. Each combined, iTRAQ-labeled tryptic digest was subjected to strong cation exchange (SCX) chromatography using a PolySulfoethyl A column and a gradient of 0-600 mM KCl in 25% acetonitrile, 10 mM KH2PO4, pH 3 with fractions collected at 1 min intervals.

Protein Identification and Quantification. SCX fractions were analyzed by online LC MS/MS with a Cap LC system and QTOF2 mass spectrometer (Waters). Protein identification utilized the Mascot search engine, and the SwissProt database. Protein quantification utilized in house code written in the statistical program R and required a minimum of two unique peptides per identified protein, ion intensities ≥ 15 for all iTRAQ tags and Mascot peptide ion scores ≥ 25 (available at http://www.lerner.ccf.org/eye/crabb/software/).

Bioinformatics Analysis. Pathways analysis was performed through Ingenuity Pathways Analysis 5.0 (http://www.ingenuity.com).

Results

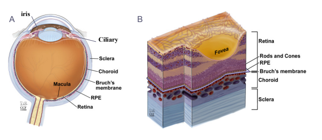


Figure 1. Uveal melanoma involves iris, ciliary body and choroid

(A) Eye anatomy; (B) Cross section showing normal Bruch's membrane/choroid complex used for the control sample.

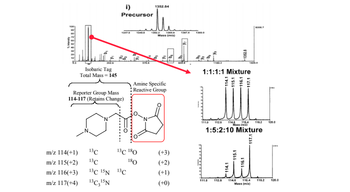
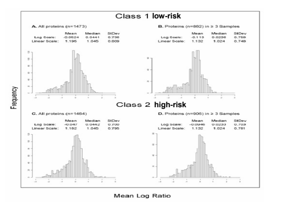


Figure 2. iTRAQ Quantitative Proteomics Technology.
iTRAQ = isobaric tags for relative and absolute quantitation

Figure 3. Distribution of Protein Ratios (Tumor/Control)



Relative protein abundance in 15 uveal melanoma tumors was determined by LC MS/MS using iTRAQ technology. Distribution of protein ratios in Log2 scale is shown for : (A) All proteins quantified in 7 class 1 low-risk tumors; (B) Proteins quantified in ≥ 3 class 1 low-risk tumors; (C) All proteins quantified in 8 class 2 high-risk tumors; (D) Proteins quantified in ≥ 3 class 2 high-risk tumors.

The protein distributions are skewed below 0, indicating generally reduced abundancy relative to the control sample.

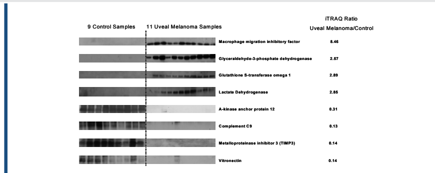


Figure 4. Western Confirmation of Quantitative Results

Western analysis of 4 elevated and 4 decreased proteins in 11 uveal melanoma and 9 control samples is shown. The Western blot results support the validity of the mass spectrometric results.

Table 3. Proteins Uniquely Abundant in Class 1 Low-Risk Uveal Melanoma Tumors

Swiss Protein Accession	Protein	Mean Ratio	SEM	p value	Donor Frequency
P08670	Vimentin	2.02	0.14	2.6E-03	7
P40926	Malate dehydrogenase, mitochondrial	2.07	0.08	9.5E-05	7
P40967	Melanocyte protein Pmel 17	1.95	0.13	3.2E-03	6
P63241	Eukaryotic translation initiation factor 5A-1	3.40	0.13	2.0E-04	6
Q13185	Chromobox protein homolog 3	2.17	0.10	6.1E-04	6
Q13510	Acid ceramidase	2.66	0.20	4.3E-03	6
O14818	Proteasome subunit alpha type-7	1.91	0.17	2.0E-02	5
P07602	Proactivator polypeptide	2.09	0.13	4.7E-03	5
P07858	Cathepsin B	2.32	0.12	2.3E-03	5
P17900	Ganglioside GM2 activator	2.45	0.04	2.2E-05	5
P20618	Proteasome subunit beta type-1	2.24	0.11	1.9E-03	5
Q14247	Src substrate cortactin	2.02	0.16	1.3E-02	5
Q9BV36	Melanophilin	2.19	0.14	5.7E-03	5
P25788	Proteasome subunit alpha type-3	2.09	0.17	2.2E-02	4
P30040	Endoplasmic reticulum protein ERp29	1.98	0.12	1.1E-02	4
P58546	Myotrophin	2.40	0.08	1.5E-03	4
P61916	Epididymal secretory protein E1	2.28	0.12	5.7E-03	4
Q9UBR2	Cathepsin Z	3.60	0.16	3.7E-03	4
P10619	Lysosomal protective protein	2.34	0.06	4.8E-03	3
P21283	Vacuolar proton pump subunit C 1	2.26	0.17	4.1E-02	3
P27695	DNA-(apurinic or apyrimidinic site) lyase	2.04	0.16	4.8E-02	3
P55809	Succinyl-CoA:3-ketoacid-coenzyme A transferase 1, mitochondrial	2.06	0.14	3.8E-02	3
Q03154	Aminoacylase-1	2.19	0.08	1.1E-02	3
Q13247	Splicing factor, arginine/serine-rich 6	2.49	0.17	3.3E-02	3
Q92820	Gamma-glutamyl hydrolase	2.38	0.06	5.6E-03	3
Q9Y2W1	Thyroid hormone receptor-associated protein 3	2.44	0.07	6.1E-03	3

Mean ratio = tumor/control; Proteins ≥ 1SD above mean; SEM = Standard error of the mean; p value from two sided t-test.

Table 4. Proteins Uniquely Abundant in Class 2 High-Risk Uveal Melanoma Tumors

Swiss Protein Accession	Protein	Mean Ratio	SEM	p value	Donor Frequency
P02787	Serotransferrin	2.35	0.14	5.5E-04	8
P08238	Heat shock protein HSP 90-beta	2.21	0.14	7.4E-04	8
P14618	Pyruvate kinase isozymes M1/M2	1.94	0.09	1.5E-04	8
P30044	Peroxiredoxin-5, mitochondrial	1.89	0.05	5.0E-06	8
P61604	10 kDa heat shock protein, mitochondrial	1.83	0.25	4.4E-02	8
P10412	Histone H1.4	2.02	0.21	1.6E-02	7
P13639	Elongation factor 2	1.95	0.10	1.3E-03	6
P25786	Proteasome subunit alpha type-1	1.94	0.15	6.5E-03	6
Q9Y4W6	AFG3-like protein 2	1.84	0.05	7.0E-05	6
O00567	Nucleolar protein 5A	1.87	0.04	9.8E-05	5
O75347	Tubulin-specific chaperone A	2.55	0.06	1.0E-04	5
P11766	Alcohol dehydrogenase class-3	2.80	0.12	1.1E-03	5
P22234	Multifunctional protein ADE2	2.25	0.04	4.5E-05	5
P46776	60S ribosomal protein L27a	1.94	0.04	4.8E-05	5
P49773	Histidine triad nucleotide-binding protein 1	2.00	0.09	1.7E-03	5
P52815	39S ribosomal protein L12, mitochondrial	2.25	0.12	2.5E-03	5
P62750	60S ribosomal protein L23a	2.38	0.11	1.4E-03	5
Q53EL6	Programmed cell death protein 4	2.64	0.08	2.4E-04	5
Q92688	Acidic leucine-rich nuclear phosphoprotein 32 family member B	1.99	0.05	1.6E-04	5
Q96KP4	Cytosolic non-specific dipeptidase	1.95	0.07	8.1E-04	5
Q9UQ80	Proliferation-associated protein 2G4	1.88	0.11	5.0E-03	5
O43399	Tumor protein D54	2.04	0.14	1.6E-02	4
P07741	Adenine phosphoribosyltransferase	2.33	0.10	3.8E-03	4
P13797	Plastin-3	2.48	0.05	4.8E-04	4
P16219	Short-chain specific acyl-CoA dehydrogenase, mitochondrial	1.92	0.13	1.6E-02	4
P24941	Cell division protein kinase 2	3.59	0.12	1.9E-03	4
P30085	UMP-CMP kinase	2.14	0.18	2.4E-02	4
P52566	Rho GDP-dissociation inhibitor 2	2.44	0.09	2.4E-03	4
Q8IV08	Phospholipase D3	2.95	0.13	3.4E-03	4
Q9BUP0	EF-hand domain-containing protein D1	1.88	0.06	2.1E-03	4
O75348	Vacuolar proton pump subunit G 1	2.40	0.09	9.8E-03	3
P08574	Cytochrome c1, heme protein, mitochondrial	2.03	0.14	3.6E-02	3
P10768	S-formylglutathione hydrolase	2.06	0.06	7.2E-03	3
P21291	Cysteine and glycine-rich protein 1	2.63	0.10	1.0E-02	3
Q13442	28 kDa heat- and acid-stable phosphoprotein	2.01	0.11	2.6E-02	3
Q15631	Translin	1.94	0.13	3.9E-02	3
Q6IS14	Eukaryotic translation initiation factor 5A-1-like	3.10	0.05	1.7E-03	3

Mean ratio = tumor/control; Proteins ≥ 1SD above mean; SEM = Standard error of the mean; p value from two sided t-test.

Table 7. Proteins Uniquely Less Abundant in Class 1 Low-Risk Uveal Melanoma Tumors

Swiss Protein Accession	Protein	Mean Ratio	SEM	p value	Donor Frequency
P32119	Peroxiredoxin-2	0.42	0.06	5.2E-06	7
P04196	Histidine-rich glycoprotein	0.41	0.26	2.7E-02	5
Q9UPT5	Exocyst complex component 7	0.41	0.04	1.8E-03	3
P0C0L4	Complement C4-A	0.38	0.09	1.6E-03	4
Q96CW1	AP-2 complex subunit mu-1	0.37	0.13	1.7E-03	5
P07358	Complement component C8 beta chain	0.37	0.09	1.5E-03	4
P12111	Collagen alpha-3(VI) chain	0.35	0.10	4.6E-05	7
P61626	Lysozyme C	0.35	0.30	4.1E-02	4
P12109	Collagen alpha-1(VI) chain	0.34	0.11	5.0E-05	7
P05787	Keratin, type II cytoskeletal 8	0.33	0.21	6.3E-03	5
P04216	Thy-1 membrane glycoprotein	0.31	0.10	7.0E-03	3
P09493	Tropomyosin alpha-1 chain	0.31	0.14	1.5E-04	7
Q15582	Transforming growth factor-beta-induced protein ig-h3	0.30	0.07	4.5E-04	4
P02751	Fibronectin	0.29	0.15	1.3E-03	5
P23229	Integrin alpha-6	0.27	0.16	1.5E-02	3
P02679	Fibrinogen gamma chain	0.24	0.13	1.0E-04	6
P60201	Myelin proteolipid protein	0.24	0.16	1.2E-02	3
P26447	Protein S100-A4	0.21	0.24	2.9E-03	5
Q01995	Transgelin	0.21	0.23	2.5E-03	5
Q92777	Synapsin-2	0.19*	0.19	1.3E-02	3
P68032	Actin, alpha cardiac muscle 1	0.17*	0.08	4.0E-06	6
Q13885	Tubulin beta-2A chain	0.16*	0.22	3.6E-03	4
Q12805	EGF-containing fibulin-like extracellular matrix protein 1	0.15*	0.03	6.8E-07	5
P07951	Tropomyosin beta chain	0.15*	0.22	3.3E-03	4
P14555	Phospholipase A2, membrane associated	0.14*	0.12	3.7E-03	3

Mean ratio = tumor/control; Proteins ≥ 1SD above mean; SEM = Standard error of the mean; p value from two sided t-test.
* Protein ratio ≥ 2SD below mean.

Table 8. Proteins Uniquely Less Abundant in Class 2 High-Risk Uveal Melanoma Tumors

Swiss Protein Accession	Protein	Mean Ratio	SEM	p value	Donor Frequency
Q9NVA2	Septin-11	0.45	0.09	2.7E-04	6
P68371	Tubulin beta-2C chain	0.45	0.06	3.1E-06	8
P05362	Intercellular adhesion molecule 1	0.45	0.07	1.3E-03	4
Q16181	Septin-7	0.45	0.07	9.0E-05	6
Q9Y490	Talin-1	0.45	0.07	3.0E-05	7
P43121	Cell surface glycoprotein MUC18	0.45	0.09	3.0E-04	6
O95782	AP-2 complex subunit alpha-1	0.44	0.19	2.4E-02	4
Q8N257	Histone H2B type 3-B	0.44	0.19	8.1E-03	6
P60033	CD81 antigen	0.44	0.10	1.4E-02	3
O94905	Erlin-2	0.43	0.08	1.2E-04	6
P07099	Epoxide hydrolase 1	0.43	0.17	1.4E-03	8
Q9NZN4	EH domain-containing protein 2	0.43	0.10	3.5E-04	6
O00468	Agrin	0.41	0.20	2.2E-02	4
Q14956	Transmembrane glycoprotein NMB	0.40	0.28	1.7E-02	7
P13987	CD59 glycoprotein	0.40	0.15	7.0E-04	7
P08571	Monocyte differentiation antigen CD14	0.39	0.26	2.1E-02	5
P29992	Guanine nucleotide-binding protein subunit alpha-11	0.38	0.14	5.9E-03	4
P35611	Alpha-adducin	0.38	0.16	2.7E-02	3
P05556	Integrin beta-1	0.38	0.09	1.3E-04	6
P02511	Alpha-crystallin B chain	0.38	0.24	5.2E-03	8
O14950	Myosin regulatory light chain MRLC2	0.36	0.05	7.5E-06	6
P06756	Integrin alpha-V	0.36	0.15	2.4E-03	5
P51648	Fatty aldehyde dehydrogenase	0.35	0.23	4.4E-02	3
O43301	Heat shock 70 kDa protein 12A	0.35	0.12	8.4E-04	5
Q9HBL0	Tensin-1	0.34	0.34	2.9E-04	5
Q14624	Inter-alpha-trypsin inhibitor heavy chain H4	0.32	0.17	2.3E-02	3
Q9UKS6	Protein kinase C and casein kinase substrate in neurons protein 3	0.31	0.15	1.5E-02	3
Q9BXN1	Asporin	0.31	0.20	4.1E-03	5
Q13425	Beta-2-syntrophin	0.31	0.08	6.5E-04	4
O43175	D-3-phosphoglycerate dehydrogenase	0.30	0.27	4.9E-02	3
Q96HC4	PDZ and LIM domain protein 5	0.30	0.18	2.1E-02	3
Q9BTV4	Transmembrane protein 43	0.28	0.22	2.2E-03	6
Q9NY15	Stabilin-1	0.28	0.27	8.9E-03	5
P07360	Complement component C8 gamma chain	0.21*	0.18	8.9E-04	5
P68366	Tubulin alpha-4A chain	0.18*	0.09	2.7E-03	3
P59768	Guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-2	0.18*	0.33	3.3E-02	3
Q2UY09	Collagen alpha-1(XXVIII) chain	0.17*	0.28	7.8E-03	4
P10643	Complement component C7	0.15*	0.36	1.3E-02	4
P63211	Guanine nucleotide-binding protein G(T) subunit gamma-T1	0.12*	0.19	7.6E-03	3

Mean ratio = tumor/control; Proteins ≥ 1SD above mean; SEM = Standard error of the mean; p value from two sided t-test.
* Protein ratio ≥ 2SD below mean.

Table 9. Networks involving Uveal Melanoma Proteins

Tumor Classification	Score	Focus Molecules	Network Pathways
Class 1	47	28	Connective Tissue Disorders, Genetic Disorder, Cellular Assembly and Organization
	44	27	Cell-To-Cell Signaling and Interaction, Tissue Development, Genetic Disorder
	37	24	Cellular Assembly and Organization, Genetic Disorder, Hematological Disease
	37	24	Genetic Disorder, Neurological Disease, Hematological Disease
	35	23	Cancer, Gastrointestinal Disease, Cell-To-Cell Signaling and Interaction
Class 2	59	33	Genetic Disorder, Hematological Disease, Organismal Injury and Abnormalities
	41	26	Cell-To-Cell Signaling and Interaction, Tissue Development, Hematological Disease
	34	23	Inflammatory Disease, Respiratory Disease, Amino Acid Metabolism
	34	23	RNA Post-Transcriptional Modification, Cellular Assembly and Organization, Cancer
	28	20	Cell Death, Hematological Disease, Hematological System Development and Function
	28	20	Cellular Assembly and Organization, Cellular Movement, Cell-To-Cell Signaling and Interaction

Bioinformatic pathways analysis was performed with Ingenuity Pathways Analysis 5.0 and all proteins elevated or decreased ± SD from the mean per tumor 1classification. Only the top scoring Networks are shown.

Table 1. Abundant Proteins in Class 1 Low-Risk Uveal Melanoma Tumors

Swiss Protein Accession	Protein	Mean Ratio	SEM	p value	Donor Frequency
P14174	Macrophage migration inhibitory factor	7.53*	0.13	4.4E-03	3
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

Mean ratio = tumor/control; Proteins ≥ 1SD above mean; SEM = Standard error of the mean; p value from two sided t-test.
* Protein ratio ≥ 2SD above mean.

Table 2. Abundant Proteins in Class 2 High-Risk Uveal Melanoma Tumors

Swiss Protein Accession	Protein	Mean Ratio	SEM	p value	Donor Frequency
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

Mean ratio = tumor/control; Proteins ≥ 1SD above mean; SEM = Standard error of the mean; p value from two sided t-test.
* Protein ratio ≥ 2SD above mean.

Table 5. Less Abundant Proteins in Class 1 Low-Risk Uveal Melanoma Tumors

Swiss Protein Accession	Protein	Mean Ratio	SEM	p value	Donor Frequency
P35579	Myosin-9	0.42	0.10	1.2E-04	7
P32119	Peroxiredoxin-2	0.42	0.06	5.2E-06	7
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

Mean ratio = tumor/control; Proteins ≥ 1SD above mean; SEM = Standard error of the mean; p value from two sided t-test.
* Protein ratio ≥ 2SD below mean.

Table 6. Less Abundant Proteins in Class 2 High-Risk Uveal Melanoma Tumors

Swiss Protein Accession	Protein	Mean Ratio	SEM	p value	Donor Frequency
Q9NVA2	Septin-11	0.45	0.09	2.7E-04	6
P00738	Haptoglobin	0.45	0.09	3.5E-03	4
P68371	Tubulin beta-2C chain	0.45	0.06	3.1E-06	8
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

Mean ratio = tumor/control; Proteins ≥ 1SD above mean; SEM = Standard error of the mean; p value from two sided t-test.
* Protein ratio ≥ 2SD below mean.

Conclusions

- For proteins quantified in all 15 uveal melanoma samples, the relative amount was greater for several proteins including macrophage migration inhibitory factor, and the relative amount was less for several proteins including TIMP3. These observations are consistent with previous results, including gene array analysis[1,2].
- Bioinformatic analysis of the significantly elevated or decreased protein components in class 1 low-risk and class 2 high-risk tumors are consistent with different biological pathways functioning in early versus late stage disease (Table 9).
- A number of proteins (n = 25-39) were found to be uniquely elevated (Tables 3 & 4) or decreased (Tables 7 & 8) in class 1 low-risk and class 2 high-risk uveal melanoma tissues. Quantitative proteomic analysis of additional uveal melanoma tissues offers promise for identifying significant biomarkers of metastasis.

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