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Analysis of the Expression and Single-Nucleotide Variant Frequencies of the Butyrophilin-like 2 Gene in Patients With Uveal Melanoma

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IMPORTANCE Chromosome 6p amplification is associated with more benign behavior for uveal melanomas (UMs) with an otherwise high risk of metastasis conferred by chromosome 3 monosomy. Chromosome 6p contains several members of the B7 family of immune regulator genes, including butyrophilin-like 2 (*BTNL2*; OMIM, 606000), which is associated with prostate cancer risk and autoimmune diseases.

OBJECTIVE To investigate the expression and variant allele frequencies of *BTNL2*, a candidate gene for chromosome 6 amplification, in patients with UM.

DESIGN, SETTING, AND PARTICIPANTS In this case-control study, we analyzed the expression of *BTNL2* in UM cell lines and human macrophages in patients with UM. Variants of *BTNL2* were analyzed using probes for polymerase chain reaction and high-resolution melting. The association of missense variants rs28362679 and rs41441651 with tumor risk was analyzed in 209 patients with UM and 116 matched control patients as well as 12 UM and 64 other tumor cell lines. Genes that were differentially expressed in M1- and M2-polarized macrophages were identified by microarray analysis of 111 patients with UM, and the association of the expression of these genes with disease-free survival was analyzed by Cox regression analysis. Data were collected from September 2013 to November 2015.

MAIN OUTCOMES AND MEASURES Butyrophilin-like 2 single-nucleotide variants were associated with UM risk; M1 and M2 macrophage-specific gene expression was associated with disease-free survival.

RESULTS We genotyped a total of 325 patients. Of the 209 patients with UM, 124 (59.3%) were male, 114 (54.5%) were Italian, and 95 (45.5%) were German; the mean (range) age was 65 (27-94) years. Of the 116 Italian control patients, 67 (57.8%) were female, and the mean (range) age was 39 (21-88) years. Butyrophilin-like 2 is expressed in patients with UM and macrophages. The frequency of the rs28362679 variant was higher in patients with UM (16 of 209 [7.7%]; 95% CI, 4.7-12.2) than frequencies from European Variation Archive and Exome Aggregation Consortium data (2134 of 118 564 [1.8%]; 95% CI, 1.7-1.9) and Exome Sequencing Project data (100 of 4540 [2.2%]; 95% CI, 1.8-2.7) but were not higher compared with Italian control patients (10 of 116 [8.6%]; 95% CI, 4.6-15.4). The rs41441651 variant was present in 5 patients with UM (2.4%; 95% CI, 0.9-5.7), 2 Italian control patients (1.7%; 95% CI, 0.1-6.5), 2846 patients from European Variation Archive and Exome Aggregation Consortium data (2.4%; 95% CI, 2.3-2.5), and 23 patients from Exome Sequencing Project data (0.5%; 95% CI, 0.3-0.8). Human UM cells express M1 and M2 macrophage-specific genes, whose expression is associated with disease-free survival.

CONCLUSIONS AND RELEVANCE Butyrophilin-like 2, expressed at various levels by UM cells and macrophages, might interfere with the immune control of the tumor. Butyrophilin-like 2 variants showed highly variable frequencies among ethnically related cohorts. There was no enrichment of *BTNL2* variants in patients with UM compared with control patients.

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Uveal melanoma (UM) is a rare malignancy, occurring in 2 to 8 people per 1 000 000, that arises from neoplastic proliferation of uveal melanocytes. A diagnosis of UM is usually made on the basis of clinical signs observed on ophthalmoscopic and imaging studies. Treatment options include local radiotherapy (eg, plaque, proton beam, or gamma knife radiotherapy) or surgical procedures (eg, local resection, endoresection, or enucleation). The 5-year survival rate is 68.9%.[1] Approximately half of patients develop distant metastases, with 90% developing cancer in the liver. The median survival of patients with metastatic UM is less than 1 year. To our knowledge, there are no adjuvant treatments and essentially no efficacious therapies for metastatic disease.[2,3]

Uveal melanoma is genetically distinct from cutaneous melanoma. The 2 diseases are characterized by different driver mutations; the *BRAF*, *NRAS*, and *NF1* genes[4] drive cutaneous melanoma whereas the *GNAQ*, *GNA11*, and *BAP1* genes[5-7] drive UM.

Considerable progress has been made in the delineation of UM multistep carcinogenesis. *GNAQ* or *GNA11* mutations, present in more than 75% of patients with UM,[5,6] are initial events that activate signaling to Yes-associated protein and transcriptional coactivator with PDZ-binding motif.[8,9] Metastasis is associated with mutations in *BRCA1*-associated protein BAP1,[7] loss of 1 copy of chromosome 3,[10,11] and amplification of chromosome 8q.[10,11] Uveal melanoma with amplification of chromosome 6p (chr6p) [10,12] tends to have a more indolent course.[11,13] To our knowledge, the cause of the attenuating effect of chr6p amplification is unknown. Since this region harbors the human leukocyte antigen complex, involvement of immune surveillance is likely. Human leukocyte antigen class I and II antigens do not appear to confer a specific genetic susceptibility.[14]

In addition to human leukocyte antigen loci, chr6p also harbors the butyrophilin-like 2 gene (*BTNL2*; OMIM, 606000), a member of the butyrophilin-like immunoregulator family[15] that shows structural and likely functional homology with the B7 family members B7-1 and B7-2 and PD-L1 and PD-L2 that bind to cluster of differentiation 28, CTLA4, and PD1.[16] CTLA4 and PD1 are 2 immunoregulatory receptors expressed on activated T effector lymphocytes, and the blocking of activated T cells has encountered major success in antitumoral therapies[17,18] with some efficacy also in patients with UM.[19-21] T-cell proliferation is repressed and cytokine release is decreased when *BTNL2* binds to an unknown receptor expressed on activated T effector cells,[15,22] suggesting the gene's role as a negative regulatory molecule for T cells. The association of *BTNL2* polymorphisms with rheumatoid arthritis, type 1 diabetes, and systemic lupus erythematosus has been attributed to the gene's strong linkage with human leukocyte antigen alleles DQ β1 and DR β1,[23] but fine-scale analyses revealed an independent association and identified a splice site mutation that abolished the membrane expression of *BTNL2* in a patient with sarcoidosis.[24] More recently, a *BTNL2* germline missense variant has been described to cosegregate with familiar prostate cancer and to be enriched in sporadic prostate cancer.[25]

Inflammation plays an important role in the progression of UM where the main infiltrating inflammatory cells are macrophages, particularly M2-polarized macrophages. In high density, M2 macrophages are associated with UM with monosomy 3.[26,27] The interference of *BTNL2* with tumor immune surveillance by either repressing pro- or antitumoral T-cell activity might be involved in the progression of UM, and overexpression after chr6p amplification or missense variation might influence UM immune surveillance. In this study, we explored the hypothesis that the effect observed for chr6p amplification in patients with UM could be attributed to *BTNL2* and its variants that are associated with prostate cancer risk. We also developed an M1- and M2-polarized macrophage signature to prognosticate UM metastasis.

Key Points

Question Does the butyrophilin-like 2 gene, an immune regulator, play a role in the risk and progression of uveal melanoma?

Findings In this case-control study, butyrophilin-like 2 was expressed in uveal melanoma tumor cells and in macrophages, especially in M2-polarized macrophages. Macrophage polarization genes were associated with uveal melanoma metastasis risk, and butyrophilin-like 2 variants showed variable frequencies among ethnically related cohorts, but no association was evident between butyrophilin-like 2 and the risk to develop uveal melanoma.

Meaning These findings suggest that inflammation plays a role in the development and progression of uveal melanoma, and butyrophilin-like 2 might play a role in the immune control of uveal melanoma and therefore could be a therapeutic target.

Methods

Patients and Samples

We genotyped a total of 325 patients. Of the 209 patients with UM, 124 (59.3%) were male, 114 (54.5%) were Italian, and 95 (45.5%) were German; the mean (range) age was 65 (27-94) years. Of the 116 Italian control patients, 67 (57.8%) were female, and the mean (range) age was 39 (21-88) years. Tissue samples and peripheral blood mononuclear cell (PBMC) samples were obtained from 209 patients with UM, including 63 with fresh frozen tissues, 19 with formalin-fixed, paraffin-embedded (FFPE) samples (7 of these patients also provided PBMC samples), and 127 with PBMC samples, and from 116 healthy control patients with PBMC samples. Patients were recruited from Ente Ospedaliera Galliera in Genova, Italy, and from the Essen University Hospital in Essen, Germany. We obtained approval from the institutional bioethics board from Ente Ospedaliera Galliera (Comitato Etico) and written informed consent from all patients. Macrodissected tumor material was conserved in RNA*later* Stabilization Solution (Ambion). For FFPE samples, two 10-μm-thick sections were cut from paraffin blocks and deparaffinized using Histoclear (Sigma-Aldrich) and rehydrated and digested overnight with proteinase K (20 μg/mL; Qiagen) at 37°C. The study was designed in July 2013, and data were collected from September 2013 to November 2015 and analyzed in December 2015.

The following UM cell lines were used: 92.1, MEL270, MEL285, MEL290, OCM1, OCM8, OMM1, OMM2.5,[28]

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MEL15765,[28] UPMD1, UPMM2, and UPMM3.[29] The DNA of 64 non-UM cell lines was obtained from the Interlab Cell Line Collection,[30] including 1301, ACN, ALL-PO, B1647, BT-474, BT-549, BV-173, BxPC3, CA46, CALU-1, CALU-6, CFPAC-1, COLO 320DMF, COLO 800, COLO 853, COR-L23, DAUDI, DMS-79, DOHH2, GDM-1, GF-D8, H9, HCT-116, Hs578T, HUP-T3, HUP-T4, IMR-32, IMR-5, IST-MEL1, IST-MEL2, IST-MEL3, IST-SL1, IST-SL2, KARPAS-422, LNCap.FGC, LoVo, LS-180, M07e, MDA-MB-231, MDA-MB-435s, MDA-MB-436, MeCo 05, MEGR07, MeMo 05, MEMOR 06, MG-63, MONO-MAC-6, OCI-AML2, PC-3, PF-382, PSN1, Rj2.2.5, SH-SY5Y, SK-LU-1, SK-MEL-24, SK-MEL-5, SK-N-AS, SK-N-BE[2], SK-N-BE[2]-C, SK-N-F1, SK-N-SH, SW48, SW480, and SW620.

Macrophages

Samples of PBMC were isolated by density gradient centrifugation over a Ficoll cushion (Histopaque 1077; Sigma-Aldrich) from platelet apheresis of healthy volunteers obtained by the Blood Transfusion Center of the Gaslini Institute in Genova, Italy, who provided written informed consent in adherence with the Declaration of Helsinki principles. Monocytes were purified using the human CD14 kit (Miltenyi Biotec), as previously described.[31] Macrophages were generated by culturing monocytes for 7 days in RPMI 1640 medium (Euroclone) supplemented with 10% fetal bovine serum (HyClone; Thermo Scientific) in the presence of 100 ng/mL of recombinant macrophage colony-stimulating factor (PeproTech; Tebu-Bio Magenta) under normoxic (20% oxygen) or hypoxic (1% oxygen) conditions. Macrophage polarization was obtained by culturing macrophages for an additional 18 hours in RPMI 1640 medium supplemented with 5% fetal bovine serum and 100 ng/mL of ultrapure lipopolysaccharide from *Escherichia coli* O111:B4 (Sigma-Aldrich) for M1 macrophages or 20 ng/mL of recombinant murine IL-4 (PeproTech) for M2 macrophages, as previously described.[32] Hypoxic conditions were obtained by cell incubation and handled in a sealed anaerobic workstation incubator (INVIVO$_2$ 400; Baker Ruskinn), incorporating a gas mixing system (Gas Mixer Q; Baker Ruskinn) and flushed with a mixture of 1% oxygen, 5% carbon dioxide, and 94% nitrogen. The medium was allowed to equilibrate in the hypoxic incubator for at least 2 hours before use.

DNA and RNA Isolation

Genomic DNA was purified from peripheral blood samples and cell lines using the QIAamp DNA blood kit (Qiagen). For FFPE tumor samples, the QIAamp DNA FFPE tissue kit (Qiagen) was used. DNA quality was assessed using NanoDrop 1000 Spectrophotometer (Thermo Scientific). RNA was extracted using the RNeasy mini kit (Qiagen).

Genotyping *BTNL2* Variants

Butyrophilin-like 2 variants rs41441651 (D336N), rs28362675 (G454C), and rs28362679 (S334L) were genotyped using commercial LightSNiP (SimpleProbe) assays from TIB-Molbiol in Berlin, Germany. Genotypes were identified with melting curve analysis of the polymerase chain reaction amplicons. The S334L and D336N variants were identified using a single LightSNiP probe and the G454C variant using its specific probe. The melting temperatures of the variants' different alleles were 57.8°C and 48.0°C for S334L, 57.8°C and 65.6°C for D336N, and 62.0°C and 54.5°C for G454C. Sanger sequencing was performed using primers 5′-TGTCAGAGAAATTGTCCAGGAAC (sense strand) and 5′-CAGACTGACCCTGCAGATAC (antisense strand).

Copy Number Analyses

Multiplex ligation-dependent probe amplification was performed (MRCHolland), as previously described.[33] Multiplex ligation-dependent probe amplification data were considered reliable if 6 or more control probes were within the normal range. Loss and gain were identified as relative ratios less than 0.7 and greater than 1.3, respectively. Array comparative genome hybridization analysis was performed using the GeneChip Mapping 250 K Assay Kit (Affymetrix), as previously described,[33] using the Copy Number Analyzer for GeneChip[34] for data analysis.

Genomic Analyses

Gene expression data for M1 and M2 genes were extracted from data set GSE5099. Preprocessing was performed in BioConductor[35] applying RMA.[36] Statistical analyses were performed using Significance Analysis of Microarray (Stanford University).[37] Uveal melanoma microarray data sets GSE22138,[38] GSE27831,[39] and GSE51880[40] were combined and normalized, as previously described.[28]

Semiquantitative real-time polymerase chain reaction was performed on oligo dT-primed complementary DNA from 1 µg of total RNA using Superscript II (RT Invitrogen). Two microliters of complementary DNA were amplified with 2.5 IU of Taq Polymerase (Thermo Fisher Scientific) using primers 5′-GTGCCATTTCCAGGATGGGA (sense strand) and 5′-CATGTGGATGCTAGGGGCAG (antisense strand). Amplification was performed on LightCycler480 II (Roche Applied Science) using 10 µL of SYBR Green Real-Time PCR Master Mix (2X) (Thermo Fisher Scientific), 0.2 µL of complementary DNA, and 0.3 µmol of primers. Expression data were normalized through the β_2-microglobulin gene expression data obtained using primers 5′-TGACTTTGTCACAGCCCAAG (sense strand) and 5′-TTCAAACCTCCATGATGCTG (antisense strand) and analyzed using Qgene.[41]

Allele Frequency Statistics

The Fisher exact test and χ^2 test were used to compare the frequency of alleles of *BTNL2* polymorphism in patients with UM with the control group and public cohorts. Hardy-Weinberg equilibrium was calculated using the calculator by Michael H. Court, PhD (https://www.coursehero.com/file/8442059/Court-lab-HW-calculator/). Statistical significance was set at $P < .05$.

Results

We analyzed the expression of *BTNL2* in UM tumor specimens, UM cell lines, and M1- and M2-polarized macrophages. **Figure 1**A shows *BTNL2* expression and amplification of chr6p for those patients for whom we had array comparative genome hybridization and expression data. Butyrophilin-like 2

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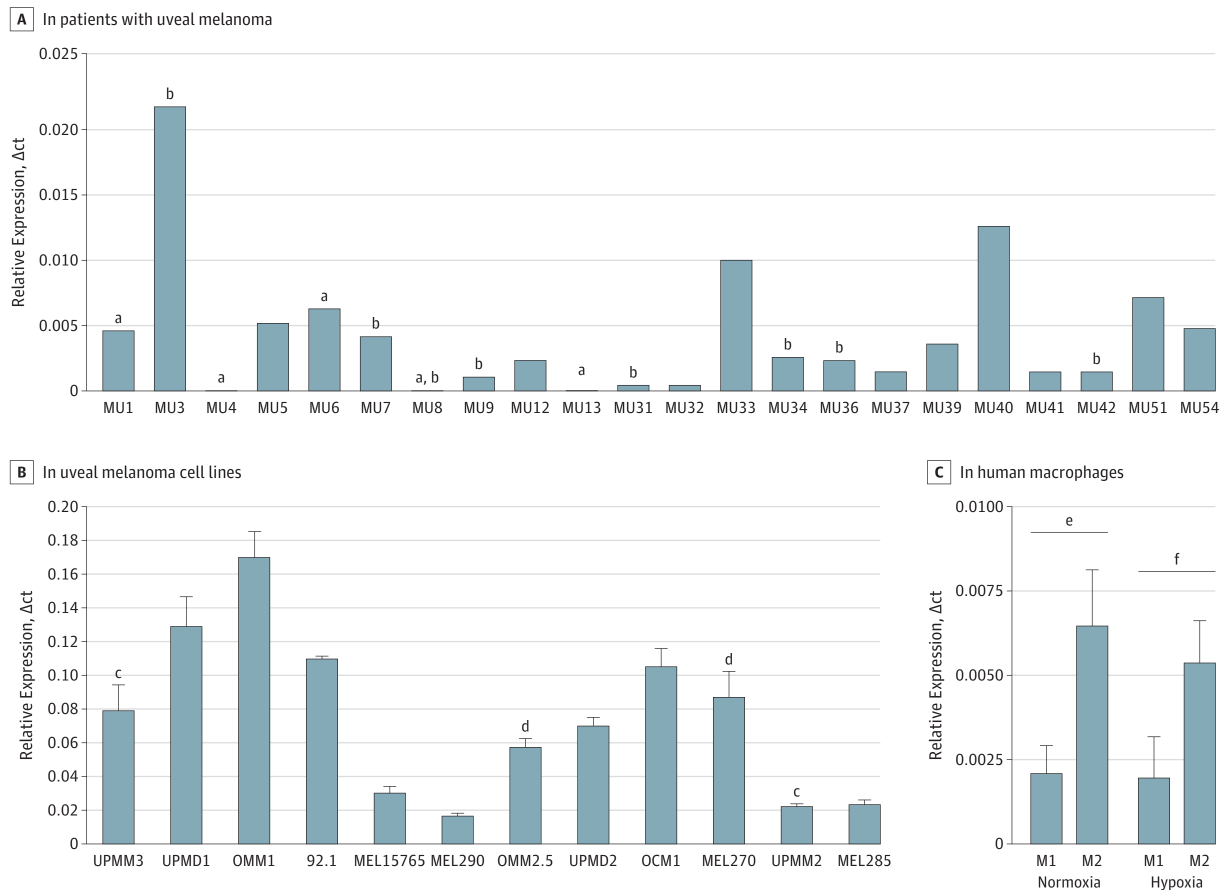
Figure 1. Butyrophilin-like 2 (*BTNL2*) Expression



A, *BTNL2* expression in patients with uveal melanoma. B, *BTNL2* expression in uveal melanoma cell lines. C, *BTNL2* expression in human macrophages generated from 5 donors. Δct indicates difference of threshold cycles. Error bars indicate standard deviation.

[a] Patients with amplification of chromosome 6.

[b] Patients who progressed to metastasis.

[c] Cell lines with monosomy of chromosome 3.

[d] Cell lines derived from the same patient.

[e] $P < .05$ by paired t test.

[f] $P < .001$ by paired t test.

is expressed at highly variable levels with no apparent association with the amplification of chr6p. The patients' MU4, MU8, and MU13 showed no *BTNL2* expression with chr6p amplification. To our knowledge, this is the first observation of *BTNL2* expression in UM tumors, although *BTNL2* expression has been reported for mucosal and lymphoid tissues.[42]

The signal obtained from human tumors may derive from the tumor cells, from the stroma, or from the tumor infiltrate. To determine whether UM tumor cells expressed *BTNL2*, different UM cell lines were analyzed. We found that UM cell lines expressed *BTNL2* at varying levels (Figure 1B). No consistent differences were observed for cell lines derived from primary and metastatic UM (ie, OMM1, OMM2.5, and MEL15765), although the primary tumor cell line MEL270 showed higher levels than the metastatic cell line OMM2.5 from the same patient.

We also analyzed *BTNL2* expression in M1- and M2-polarized macrophages, generated from peripheral blood monocytes. Butyrophilin-like 2 expression was detected in both M1 and M2 macrophages and was significantly higher in the M2 subtype independent from oxygenation (Figure 1C), suggesting the gene's potential contribution to the M2 immunosuppressive and tumor-promoting activity. Exposure to hypoxia slightly decreased *BTNL2* expression in M2 but not in M1 macrophages, suggesting that the hypoxic microenvironment is not a critical regulator of *BTNL2* in macrophages.

Because *BTNL2* expression in the tumor as well as in macrophages might contribute to tumor progression, we wished to determine whether prostate cancer risk-associated single-nucleotide variants (SNVs) of this gene were also enriched in patients with UM. We analyzed 209 patients, including 114 from Genoa and Bergamo, Italy, and 95 from Essen, Germany, as well as 116 healthy control patients from Italy. The variants reported to be associated with prostate cancer risk, D336N and G454C, were completely linked in all 134 patients that we analyzed for both SNVs. The LightSNiP assay for D336N also revealed the nearby S334L variant that was associated with alopecia.[43] The LightSNiP assay was conducted so that the probe yielded clearly distinct melting peaks for the 2 SNVs and the

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Table. Allele Frequencies for *BTNL2* Variants rs41441651 and rs28362679

Sample	Total Patients, No.	Patients With rs28362679 Variant, No. (%)	Frequency, % (95% CI)	*P* Value[a]	Patients With rs41441651 Variant, No. (%)	Frequency, % (95% CI)	*P* Value[a]
Patients with UM	209	16 (7.7)	7.7 (4.7-12.2)	[Reference]	5 (2.4)	2.4 (0.9-5.7)	[Reference]
Italian patients	114	9 (7.9)	7.9 (4.1-14.6)	NA	4 (3.5)	3.5 (1.1-9.0)	NA
German patients	95	7 (7.4)	7.4 (3.4-14.8)	NA	1 (1.1)	1.1 (0.0-6.4)	NA
Control patients	116	10 (8.6)	8.6 (4.6-15.4)	.83	2 (1.7)	1.7 (0.1-6.5)	>.99
Cohort							
EVA/EXAC (worldwide population)	118 564	2134 (1.8)	1.8 (1.7-1.9)	<.001[b]	2846 (2.4)	2.4 (2.3-2.5)	>.99
ESP cohort (North America population)	4540	100 (2.2)	2.2 (1.8-2.7)	<.001	23 (0.5)	0.5 (0.3-0.8)	.007
1000 Genomes database							
Europeans	1008	16 (1.6)	1.6 (1.0-2.6)	<.001	3 (0.3)	0.3 (0.1-0.9)	.005
Americans	694	9 (1.3)	1.3 (0.7-2.5)	<.001	9 (1.3)	1.3 (0.7-2.5)	.33
Cell lines							
UM	12	0	0.0 (0-28.7)	NA	2 (16.7)	16.7 (3.8-46.2)	NA
Non-UM tumor	82	3 (3.7)	3.7 (0.9-10.8)	NA	1 (1.2)	1.2 (0.0-7.4)	NA

Abbreviations: *BTNL2*, butyrophilin-like 2, ESP, Exome Sequenicng Project; EVA, European Variation Archive; EXAC, Exome Aggregation Consortium; NA, not applicable; UM, uveal melanoma.

[a] *P* value derived from Fisher exact test.

[b] *P* value derived from χ^2 test.

wild-type allele. The presence of both SNVs together with the wild-type allele in the same sample would have had 3 distinct peaks; because this did not happen, we can conclude that D336N and S334L were never observed together in our patients. Therefore, in our samples, 3 genotypes were observed: (1) all 3 variants with the major wild-type allele, (2) D336N and G454C with the minor allele and S334L with the wild-type allele, and (3) D336N and G454C with the wild-type allele and S334L with the minor allele. MEL270 and OMM2.5 UM cells, derived from the same patient, were heterozygous, and the pancreas cell line PSN1 was homozygous for D336N. S334L was not found in the cell lines analyzed.

The allele frequencies of the single SNVs observed in patients with UM were compared with allele frequency data reported in the publicly available cohorts, including the European Variation Archive and Exome Aggregation Consortium (worldwide population), Exome Sequencing Project (North America population), 1000 Genomes database Europeans, and 1000 Genomes database Americans (**Table**). S334L was present in 16 of 209 patients with UM (7.7%; 9 of 114 Italian patients [7.9%]; 95% CI, 4.1-14.6; 7 of 95 German patients [7.4%]; 95% CI, 3.4-14.8) and in 10 of 116 healthy Italian control patients [8.6%]. Published cohorts reported different frequencies of 1.8% to 2.2%.[44] D336N was present in 5 patients with UM (2.4%; 4 Italian patients [3.5%] and 7 German patients [7.4%]) and in 2 Italian control patients (1.7%; *P* = .44). Data from 2 public cohorts, the Exome Sequencing Project and 1000 Genomes database Europeans, show different frequencies (0.5% and 0.3%, respectively). European Variation Archive and Exome Aggregation Consortium data show exactly the same frequency (2.4%) as our patients with UM, and 1000 Genomes database Americans data show a different frequency (1.3%). No deviation from Hardy-Weinberg equilibrium was observed for either variant in patients and controls.

Despite the absence of a clear association between *BTNL2* variants and UM risk, expression of this immunoregulator might affect the proinflammatory infiltrate in patients with UM.[26] The presence of a high number of infiltrating macrophages with M2-like phenotype, considered to be proangiogenic and tumor promoting, is associated with a bad prognosis.[27] Therefore, we extended our analysis to the expression of M1 and M2 genes in 111 patients with UM from our data[28,40] and from the data of Laurent et al.[38] We created a list of genes associated with M1- and M2-polarized macrophages starting from raw data of M1- and M2-polarized macrophages reported by Martinez et al[32] (data set GSE5099). We identified 1280 significantly differentially expressed genes (M1 and M2 genes) using the Significance Analysis of Microarray bootstrapping algorithm[37] (eTable 1 in the Supplement). We then extracted the expression values of these genes from the UM data sets and analyzed which of the M1 and M2 genes were significantly differentially expressed in metastatic vs nonmetastatic patients with UM (**Figure 2**). Metastatic and nonmetastatic samples show differential expression of 102 of the M1 and M2 polarization-specific genes (eTable 2 in the Supplement) that we used for hierarchical clustering analysis. Two main clusters were formed, one of which was enriched for metastatic samples. We then calculated a multigene score (MGS) by a forward stepwise likelihood ratio analysis. The MGS is formed by the sum of the gene expression values multiplied by the respective multivariate β coefficient ($E_{g1}\beta_{g1} + E_{g2}\beta_{g2} + \ldots + E_{gn}\beta_{gn}$). The calculated MGS contains only 6 genes whose expression values were independently associated with metastasis-free survival (ie, cathepsin C, tyrosylprotein sulfotransferase 1, guanosine monophosphate reductase, mannosidase α class 2A member 1, metadherin, and calcium homeostasis modulator 2). We applied the MGS to classify patients with UM according to the median value of MGS and calculated Kaplan-Meier survival curves (**Figure 3**). The score yielded a highly significant classification (*P* < .001) of high- and low-risk samples. Under the assumption that the gene expression signals were mainly derived from the inflammatory infiltrate, the results of this analysis demonstrated that inflammation plays a role in the formation and progression of UM.

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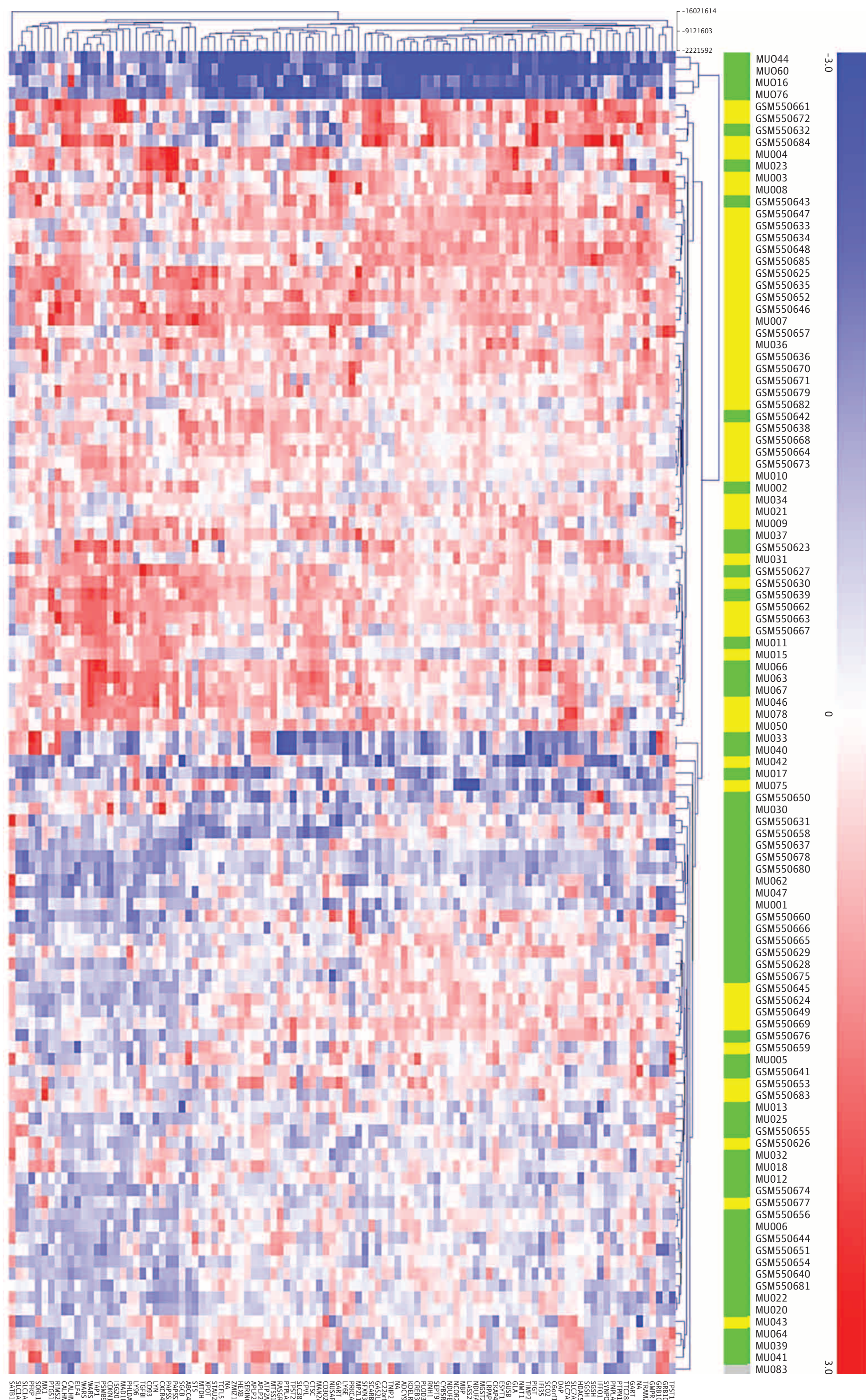
Figure 2. Expression of M1- and M2-Polarized Macrophage-Specific Genes in Uveal Melanoma Samples



Hierarchical clustering shows 2 gene clusters with differential expression relative to the expression data for each single gene in all samples analyzed; blue indicates the value is below the mean, red indicates it is above the mean, and white indicates it is at the mean. One cluster is enriched for patients who progressed to metastasis; green indicates metastasis and yellow indicates no metastasis.

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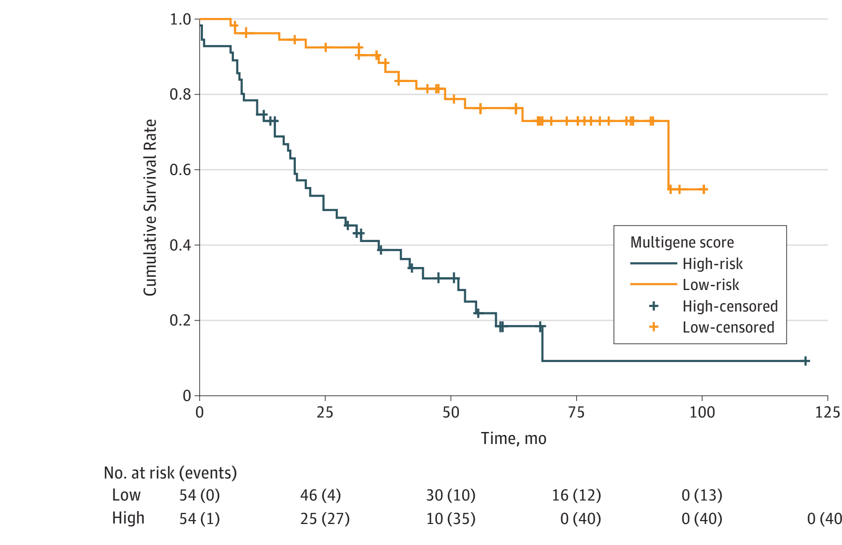
Figure 3. Survival According to the M1- and M2-Polarization Multigene Score



A multigene score was calculated by Cox regression analysis using differentially expressed genes, and samples were classified according to the median value of the multigene score in high-risk (blue) and low-risk (orange) patients.

Discussion

To identify potential mediators of prometastatic inflammation in patients with UM, we analyzed *BTNL2*, given its negative regulatory role on T-cell functions in inflammatory and autoimmune diseases.[22] Butyrophilin-like 2 is located on chr6p and has been described to be associated with the risk of familiar and sporadic prostate cancer.[25] Butyrophilins are members of the B7 immunoregulator family, believed to control T-cell activation.[45] Antibodies against CTLA4 and PD1 that are expressed by inflammatory and tumor cells[17,18] act by inhibiting CTLA4 and PD1 from binding to B7-1 and B7-2 and PD-L1 and PD-L2 molecules, respectively. Given the success of these antibodies in the clinics,[16,17] butyrophilins and butyrophilin-like proteins are interesting targets for additional immune therapies. Butyrophilin-like 2 has been described to inhibit the proliferation of murine CD4$^+$ T cells and to reduce the proliferation and cytokine production of activated T cells,[22] with implications for tumor progression. Butyrophilin-like 2 has also been shown to induce forkhead box P3 expression and regulatory T-cell differentiation, thus maintaining an immunosuppressive environment at mucosal surfaces.[46] The expression of *BTNL2* by both UM tumor cells and macrophages suggests *BTNL2* should be a candidate for further investigation. We also addressed hypoxia, an important regulator of monocyte and macrophage functions in the tumor microenvironment,[47] which did not show major effects on *BTNL2* expression.

Several genetic variants and their associations with cancer risk[25] and other conditions[24,43] have been previously described for *BTNL2*. D336N, an A>G variant, determines the substitution of an aspartic acid with asparagine in position 336 in the extracellular portion in an immunoglobulin-like variable 3 domain of BTNL2 protein. The substitution is classified as “benign,” with a score of 0.092—where 0 indicates absolutely unlikely to affect protein function and 1 indicates certainly affecting protein function—by Polyphen2 prediction of functional effects of human nonsynonymous SNVs.[48] G454C, a C>G variant, determines the substitution of a glycine residue acid with arginine in position 454, the second-to-last amino acid of *BTNL2*. Given the location of the latter variant on the tip of the extracellular domain that must interact with T and B cells and the vascular endothelium,[22,45,46] functional consequences are likely. The substitution is classified as “possibly damaging,” with a score of 0.894 by Polyphen2. S334L, a C>T variant, determines the substitution of a serine residue with leucine in position 334. The substitution is classified as “benign,” with a score of 0.247 by Polyphen2.

We observed a strong discrepancy between our healthy control patients and the public cohorts that cannot be attributed to different ethnic compositions of the cohorts, since our analysis included German and Italian patients who were expected to account for a significant part of the Exome Sequencing Project cohort of North Americans and of the 1000 Genomes database Europeans cohort. Based on the analysis of our patients and control participants, the 2 *BTNL2* variants do not appear to be associated with risk to develop UM. Next-generation sequencing (public cohorts) and LightSNiP polymerase chain reaction (our data) are different technical approaches but have similar sensitivities and specificities. There is no reason to attribute the different frequencies between our control patients and these cohorts to the analysis techniques used. Therefore, the differences remain unexplained and should be taken into account in similar studies on these SNVs.

This study has some limitations inherent in observational case-control studies. We only analyzed Italian and German patients and Italian control patients. Including patients and controls from other European countries would have allowed us to match the composition of the exome sequencing cohorts and to determine the true frequencies of the variants analyzed.

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Conclusions

The expression of *BTNL2* by UM tumor cells and macrophages hints at a potential role in the gene's control of tumor progression through the immune system, but there were no apparent associations between tumor progression and chr6p amplification or prostate cancer–associated variants. Future research should consider the role of *BTNL2* expression in both UM cell and macrophages for controlling UM.

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Invited Commentary

Genetic Variants of the *BTNL2* Gene in Uveal Melanoma

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Significant progress has been made in understanding the molecular pathology of uveal melanoma (UM). It is well known that genetic alterations, such as monosomy 3, polysomy 8q, and *BAP1* gene-inactivating mutations, are associated with a poor prognosis in UM, whereas a gain in chromosome 6p is associated with a more favorable outcome.[1] However, the pathways by which these genetic aberrations influence the processes involved in tumor dissemination and ultimate colonization are not fully understood.

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In this issue of *JAMA Ophthalmology*, Amaro et al[2] present an extensive analysis of the *BTNL2* gene in UM and its association with macrophage infiltrates in these tumors. Chromosome 6p harbors the *BTNL2* gene, which is a member of the butyrophilin-like B7 family of immunoregulators.[3] *BTNL2* gene polymorphisms have been implicated in a number of diseases such as sarcoidosis, rheumatoid arthritis, inflammatory bowel disease, type 1 diabetes, and systemic lupus erythematosus. It has also been associated with prostate cancer.[4] To our knowledge, the role of this gene has not yet been studied in UM. It is believed that the gene may be involved in immune surveillance as a negative T-cell regulator by decreasing T-cell proliferation and cytokine release, which would be protumor progression.[3,5,6] Hence, it would seem to be contradictory to the more indolent course associated with a chromosome 6p gain in UM.

Amaro et al[2] investigated the expression and missense variant frequencies of the *BTNL2* gene in UM samples from patients treated in Italy and Germany, UM cell lines as well as in human macrophages (after in vitro polarization into M1 and M2 subsets) by real-time polymerase chain reaction and multiplex ligation-dependent probe amplification. They found that *BTNL2* was expressed in UM specimens and UM cell lines at highly variable levels with no correlation with the amplification of chromosome 6p. It should be noted that not all of the examined UM samples were demonstrated in the Results section; the authors only analyzed those with aCGH data. Interestingly, there was also no difference seen in cell lines derived from primary or metastatic UM. The authors also demonstrated that there was no correlation between the frequencies of missense variants with UM risk. Furthermore, no

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