Genetic Risk Factors for Radiation Vasculopathy

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Uveal melanoma, the most common primary intraocular malignancy, is routinely treated by irradiation. Proton beam irradiation (PBI) has been successfully used at the Massachusetts Eye and Ear (MEE) since 1975, with local control rates approaching 95% at 15 years after treatment.1 However, radiotherapy may lead to significant visual loss, particularly when the tumor is located near the optic nerve or macula. Radiation injury results in a microangiopathy secondary to endothelial cell loss and capillary closure, which is most visually significant when it affects the capillaries of the macula or optic nerve. We previously demonstrated that the majority of patients with tumors located within 4 disc diameters (DD) of the optic nerve or macula experienced radiation complications (35% developed papillopathy and 64% developed maculopathy).2 In patients who developed radiation complications, 35% developed papillopathy and 64% developed maculopathy, respectively. In patients with melanomas located within 1 DD of the optic nerve but without foveal involvement, papillopathy occurred in 68%.3 More recently, we reported that 35.5% of patients with tumors within 1 DD of the fovea retain 20/200 or better vision 5 years after treatment with PBI.4 However, 20% of patients with macular tumors within 1 DD of the fovea with height less than 5 mm retained 20/40 vision or better 5 years after irradiation. Therefore, there is a minority of patients that retain good vision after PBI even if the tumor is located close to the nerve or the macula.

It is not understood why some patients retain good vision years after PBI despite receiving high doses of radiation to the fovea and/or optic nerve. A study of individual variation in the development of skin telangiectasia after bilateral internal mammary irradiation suggested that patient-related factors accounted for 81% to 90% of the variation in this response to radiation.5 The study of “radiogenomics” attempts to elucidate the genetic factors that may influence individual susceptibility to the adverse effects of radiotherapy.6

Investigators have previously reported single nucleotide variation that confers susceptibility to radiation in various types of cancers. Single nucleotide polymorphisms (SNPs) in the genes ATM (detection of DNA damage), SOD2 (scavenging of reactive oxygen species), XRCC1 (DNA repair), XRCC3 (DNA

PURPOSE. The purpose of this study was to perform a genome-wide scan for polymorphisms associated with risk of vision loss from radiation complications in patients treated with proton beam irradiation for choroidal melanoma.

METHODS. We identified a cohort of 126 patients at high risk of radiation complications due to tumor location within 2 disk diameters of the optic nerve and/or fovea who provided a blood sample to the Massachusetts Eye and Ear Uveal Melanoma Repository. Controls (n = 76) were defined as patients with visual acuity 20/40 or better 3 years after treatment. Cases (n = 50) were selected as patients with visual acuity 20/200 or worse due to radiation damage 3 years after treatment. Genotyping of these samples was performed using the Omni 2.5 chip (Illumina, Inc.).

RESULTS. Hypertension (odds ratio [OR] = 3.749, P = 0.0009), visual acuity at diagnosis of choroidal melanoma (OR = 1.031, P = 0.0022), tumor distance to fovea (OR = 0.341, P = 6.52E-05), tumor distance to optic disc (OR = 0.481, P = 5.41E-05), and height of tumor (OR = 1.704, P = 0.0069) were associated with poor vision (20/200 or worse). Individual single nucleotide polymorphism (SNP) analysis was performed controlling for the risk factors identified using stepwise regression and the first principal component. Although this analysis determined that there were 74,529 nominally significant SNPs (P < 0.05), there were no SNPs that reached genome-wide significance (P < 5E-08). The SNP reaching the highest significance level (P < 1E-04) was rs11678387, located on chromosome 2, intergenic between EPB41L5/RALB (P = 4.43E-05).

CONCLUSIONS. Visual loss from radiation vasculopathy after treatment for choroidal melanoma is not only related to tumor location but may be influenced by hypertension and possibly genetic factors.

Keywords: radiation damage, GWAS, proton beam irradiation, SNP analysis, choroidal melanoma
repair), and TGFβ1 (tissue remodeling) have been associated with radiation toxicity in patients treated with radiotherapy for several forms of cancer.7

We performed this study to identify potential genetic risk factors that influence vision loss from radiation vasculopathy in patients receiving PBI for choroidal melanoma close to the optic nerve or the macula.

METHODS

We identified a cohort of 126 unrelated patients at high risk of radiation complications due to tumor location within 2 DD of the optic nerve and/or fovea who provided a blood sample to the Massachusetts Eye and Ear Uveal Melanoma Repository. Only patients with tumors <5 mm in height and <15 mm in diameter were included. Patients with larger tumors were excluded because factors other than radiation-induced complications can contribute to vision loss in these cases. Controls (n = 76) were defined as patients with visual acuity 20/40 or better 3 years after treatment (Fig. 1). Cases (n = 50) were selected as patients with visual acuity 20/200 or worse due to radiation papillopathy or retinopathy 3 years after treatment (Fig. 2). No patient in this cohort received pharmacologic therapy for radiation vasculopathy. For a schematic of the study design, please refer to Figure 3. The majority (69.8%) of patients received a radiation dose of 70 Gy (relative biological effectiveness [RBE]). The remaining patients received a dose of 50 Gy (RBE), reflecting a change in the standard protocol followed for patients with small or medium tumors located near the optic nerve or macula.

The cohort of 126 individuals was genotyped using Illumina’s HumanOmni2.5-8 BeadChip v1.2 (San Diego, CA, USA). Epidemiologic variables such as sex, age, hypertension, diabetes, and tumor details were recorded for each subject. SNP data cleaning and analysis was performed using PLINK8 (http://pngu.mgh.harvard.edu/~purcell/plink/, provided in the public domain). Data cleaning was performed using standard procedures.9 Specifically, SNPs with a low genotyping pass rate (>10% of genotypes missing from the entire cohort), SNPs with only one or less minor alleles observed (minor allele frequency <0.3%), and/or SNPs that were not in Hardy-Weinberg equilibrium (HWE) in control subjects (HWE P < 0.0001) were removed. Individuals were removed that had >5% of genotypes missing. Heterozygosity rates across samples were checked and outlier samples were excluded. Data was checked for sex discrepancies and cryptic relatedness. Assessment of population substructure in this dataset was performed using principal components analysis (PCA) with Eigensoft10,11 (http://www.hsph.harvard.edu/alkes-price/software/, provided in the public domain).

Epidemiologic variables were tested for their association with poor visual acuity (20/200 or worse) using logistic regression in SAS (SAS Institute, Cary, NC, USA). Stepwise logistic regression was performed to determine the most significantly associated epidemiologic risk factors. For the SNP analysis, the minor allele, or less frequent allele, for each SNP was tested for association with poor visual outcomes using
logistic regression in PLINK. Association tests were performed controlling for the significant epidemiologic factors resulting from the stepwise regression, along with the significant principal components (PCs). Correction for multiple testing was performed using genomic control (GC), Bonferroni, and false discovery rate (FDR). Tests for gene–gene interaction were performed on the top hits using the epistasis function in PLINK. To determine whether the combination of SNPs within a given gene was more significantly associated with the outcome than with any single SNP alone, gene-based tests were performed using set-based tests in PLINK. Briefly, for each gene, test statistics were determined by calculating the mean of each independent signal within the gene. The dataset was then permuted 10,000 times, keeping linkage disequilibrium (LD) between SNPs constant and varying the phenotype labels. Bioinformatic analyses were performed using the commercially available Ingenuity Pathway Analysis (IPA; http://www.ingenuity.com/products/ipa, provided in the public domain), and freely available data for e-QTL analysis from the website SCANdb (http://www.scandb.org/newinterface/about.html, provided in the public domain) and for microRNA analysis from freely available websites miRDB (http://www.mirdb.org/mirdb/, provided in the public domain) and TargetScan (http://www.targetscan.org/vert_71/, provided in the public domain).

The study was approved by the Massachusetts Eye and Ear Institutional Review Board. The research study followed the tenets of the Declaration of Helsinki, and informed consent was obtained from the subjects after explanation of the nature and possible consequences of participating in the repository.

### RESULTS

#### Baseline Characteristics

Analysis of baseline factors revealed that both groups were well matched in terms of radiation dose, age, sex, and presence of diabetes. The majority of both controls and cases received 70 Gy (RBE) (69.7% and 70.0%, respectively; $P = 1.0$). The control group (good vision) had a lower rate of hypertension at baseline (21% vs. 50%, $P = 0.001$) and tumors located farther away from the disc (median, 2 vs. 0.75 mm; $P < 0.0001$) and fovea (median, 1.2 vs. 0 mm; $P < 0.0001$) compared with the cases (poor vision; Table 1).

#### Factors Associated With Poor Vision

Epidemiologic variables were tested for association with poor visual acuity (20/200 or worse): age, sex, diabetes, hypertension, visual acuity at diagnosis of choroidal melanoma, tumor distance to macula, tumor distance to optic disc, height of tumor, and largest basal diameter. Of these variables, hypertension (odds ratio [OR] = 3.749, $P = 0.0009$), visual acuity at diagnosis of choroidal melanoma (OR = 1.031, $P = 0.002$), tumor distance to macula (OR = 0.341, $P = 6.52E-05$), tumor distance to optic disc (OR = 0.481, $P = 5.41E-05$), and height of tumor (OR = 1.704, $P = 0.0069$) were associated with poor visual acuity (20/200 or worse). The stepwise regression model showed that tumor height, tumor distance to optic disc, tumor distance to macula, and hypertension were the most significantly associated risk factors after adjustment for each other variable (Table 2). The dose of radiation (70 vs. 50 Gy) was not associated with poor visual acuity.

<table>
<thead>
<tr>
<th>Table 1. Baseline Characteristics</th>
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<tbody>
<tr>
<td><strong>Patient/Tumor Characteristics</strong></td>
</tr>
<tr>
<td>Age at treatment, y (median, range)</td>
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<tr>
<td>Sex, male (n, %)</td>
</tr>
<tr>
<td>Diabetes at baseline (n, %)</td>
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<tr>
<td>Hypertension at baseline (n, %)</td>
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<tr>
<td>Distance to the optic disc, DD (median, range)</td>
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<tr>
<td>Distance to the fovea, DD (median, range)</td>
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<td>Tumor location within 2 DD of both fovea and optic disc (n, %)</td>
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<tr>
<td>Largest basal diameter, mm (median, range)</td>
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<tr>
<td>Height, mm (median, range)</td>
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<tr>
<td>Baseline visual acuity (median, range)</td>
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<tr>
<td>Radiation dose of 70 Gy (n, %)</td>
</tr>
</tbody>
</table>
TABLE 2. Factors Associated With Poor Visual Acuity (20/200 or Worse)

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>OR</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypertension</td>
<td>5.749</td>
<td>1.715-8.194</td>
</tr>
<tr>
<td>Visual acuity at diagnosis</td>
<td>1.051</td>
<td>1.011-1.051</td>
</tr>
<tr>
<td>Tumor distance to fovea</td>
<td>0.341</td>
<td>0.201-0.578</td>
</tr>
<tr>
<td>Tumor distance to optic nerve</td>
<td>0.481</td>
<td>0.337-0.686</td>
</tr>
<tr>
<td>Height of the tumor</td>
<td>1.704</td>
<td>1.158-2.507</td>
</tr>
</tbody>
</table>

SNP Analysis
A total of 2,338,671 SNPs were genotyped in 126 individuals. In data cleaning, no individuals were removed from analysis due to low genotyping rates, sex discrepancies, or cryptic relatedness. There were 769,004 SNPs removed during data cleaning (501 markers failed HWE test, 18,575 SNPs failed missingness test, and 753,956 SNPs had less than one minor allele in the entire cohort, therefore failing the frequency test), leaving 1,569,667 analysis-ready SNPs.

Single SNP analysis was performed controlling for the risk factors identified using stepwise regression and the first PC. Although this analysis determined that there were 74,529 nominally significant SNPs (P < 0.05), there were no SNPs that reached genome-wide significance (P < 5E-08; Fig. 4).

The SNPs reaching the highest significance level (P < 1E-04) were rs11678387, an intergenic SNP located on chromosome 2 (P = 4.43E-05) shown to increase risk of poor vision; rs8133945, an intergenic SNP located on chromosome 21 (P = 6.67E-05) shown to decrease risk of poor vision; and rs16921196, a SNP located on chromosome 8 in the gene JMJD2D (lysine demethylase) in the CEU (Utah Residents with Northern and Western European Ancestry) population. In the YRI (Yoruba in Ibadan, Nigeria) population, this SNP was shown to alter expression of the gene AMN (amnion associated transmembrane protein). The other two SNPs, rs8133945 and rs16921196, were not shown to be e-QTLs for any other genes.

Using the two microRNA databases, we found that none of the significant SNPs were predicted to be within a microRNA or within a microRNA binding site.

As a result, the top two SNPs, rs11678387 and rs8133945, were predicted to have disease risk for radiation retinopathy. The third most significant SNP, rs16921196, was not shown to be e-QTLs for any other genes.

Bioinformatic Analysis
Using SCANdb, we investigated the most significant SNPs for expression–quantitative trait loci (e-QTL). The chromosome 2 SNP rs11678387 was shown to alter the expression of the genes MAP3K7IP3 (MAP3 kinase interacting protein 3), SYT11 (synaptotagmin 11), PCNL2 (pecanex-like protein 2), and JMJD2D (lysine demethylase) in the CEU (Utah Residents with Northern and Western European Ancestry) population. In the YRI (Yoruba in Ibadan, Nigeria) population, this SNP was shown to alter expression of the gene AMN (amnion associated transmembrane protein). The other two SNPs, rs8133945 and rs16921196, were not shown to be e-QTLs for any other genes.

Pathway analysis using the set of SNPs showing nominal significance (P < 0.05) in this dataset showed that the most common canonical/classical pathways involving the genes in which these SNPs are located were as follows: axonal guidance signaling, sperm motility, cellular effects of Sildenafil, endothelin-1 signaling, and neuropathic pain signaling in dorsal horn neurons. The diseases and disorders most common within these genes were cancer, dermatologic diseases and conditions, organinal injury and abnormalities, gastrointestinal disease, and reproductive system disease.

DISCUSSION
Radiation retinopathy remains a significant cause of visual morbidity in patients with choroidal melanoma undergoing PBI. We previously identified radiation dose to the optic nerve and the macula as the main risk factor for radiation papilopatphy and maculopathy respectively. Additionally, we showed that a reduction of the dose from 70 to 50 Gy (RBE) did not seem to increase the proportion of patients that retain
useful vision. In this study, we identified hypertension, distance to the optic nerve and/or the macula, height of the tumor, and vision prior to radiation as being risk factors associated with poor vision at 3 years after PBI. Total dose received (70 vs. 50 Gy) was confirmed again in this study to not be associated with poor visual acuity.

Despite the careful phenotypic selection of cases and controls in this study, we were unable to identify any SNP that reached genome-wide significance in terms of association with risk of vision loss from radiation complications. Considering our most significant result, we still had 60% power to detect our strongest association, rs11678387, given an OR > 10^-08. This study was slightly underpowered (60% vs. 80%) to detect a genome wide level of association at 10^-08; however, it is interesting to consider the top hits from this genome-wide association study (GWAS) in terms of potential biologic relevance.

The SNP reaching the highest significance level (P < 1E-04) was rs11678387, an intergenic SNP located on chromosome 2 (P = 4.43E-05) shown to increase risk of poor vision. It occurs between EPB41L5 and RALB. Jensen et al. showed that the zebrafish gene EPB41L5 is expressed in the RPE and that mutations in the gene produce retinal lamination defects. Jensen and Westerfield found that mutant fish also show defects in the kidney and brain, including edema and small or absent brain ventricles. They also proposed that EPB41L5 is required for tight junction formation in the RPE and is part of the pathway that controls apical cell polarity in photoreceptor morphogenesis, as well as in other epithelial-derived tissues. It is of interest to mention that different SNPs within the EPB41L5 gene were identified as the 6th, 11th, and 13th most significant hits in our cohort. However, gene-based analysis for this gene did not reach significance.

RALB is one of two isoforms of the Ral protein, the other being RalA, and part of the Ras GTPase family. Ral proteins have been associated with the progression of several cancers, including bladder cancer and prostate cancer. Depletion of RalB has been shown to increase the stability and half-life of p53, which is a major regulator of the cellular response to DNA damage, including expression of DNA repair genes, and induction of senescence and apoptosis. Such mechanisms are certainly relevant to response to neural and endothelial cells in the retina and optic disc to radiation damage.

The next most significant SNP is located on chromosome 21 (P = 6.67E-05) and was shown to decrease risk of poor vision. It is located between the genes DSCAM and BACE2 on chromosome 21. DSCAM is a transmembrane protein with a very high structural and sequence homology to the immunoglobulin superfamily of cell adhesion molecules. It is expressed in the developing nervous system with the highest level of expression occurring in the fetal brain. When this gene is knocked out in mice, a relatively normal neural retina is produced, but the brain and kidneys exhibit defects. They also proposed that DSCAM is involved in the development of the nervous system. In addition, DSCAM is a member of the Ig-superfamily and plays a role in cell adhesion. DSCAM is also expressed in pigmented cells and it cleaves amyloid fibrils. Although it is highly expressed in RPE and choroid, its function is unknown. Assessment of the bace1-/- and combined bace1-/- bace2-/- knockout mice demonstrated a significantly reduced retinal and choroidal vascular density, retinal thinning, apoptosis, thinning of Bruch’s membrane, and a greater than twofold increase in lipofuscin in the RPE. BACE2-/- mice exhibited a relatively normal neural retina.
although occasional foci of neural retinal hyperplasia are observed. These animals exhibited a highly disrupted choroid together with swelling and lipofuscin accumulation in the overlying RPE, but these changes are less severe than in BACI/ + animals.19

The third most significant SNP, rs16921196, is located in intron 1 of the gene XKR4. Its function has not yet been characterized.

Pathway analysis identified functions (axonal guidance signaling, sperm motility, cellular effects of Sildenafil, endothelin-1 signaling, and neuropathic pain signaling in dorsal horn neurons) that have potential links to the disease process under investigation. Axonal guidance signals have been shown to be important in regeneration of retinal ganglion cell axons after optic nerve injury.20 Therefore, this function may also determine responses to optic nerve or retinal injury from radiation. Similarly, pathways involved in sperm motility involve calcium and CAM signaling, reactive oxygen species, and mitochondrial function.21 These pathways also have relevance to cellular responses to radiation. In addition, pathways related to vascular tone emerged that may influence vascular damage secondary to radiation.

It is important to note that the three most significant SNPs in this study are all noncoding variants. The first two are intergenic and the third one intronic. The regulatory function of noncoding sequences continues to be elucidated. These portions of the genome include transcriptional silencers or enhancers, modifiers of chromatin structure, and gene methylation regulators.22 Therefore, it is certainly possible that the functional role of these SNPs may not involve the genes encoded in proximity to these variants, but rather effects on other proteins encoded elsewhere in the genome.

Six GWAS studies exploring genetic risk factors in normal tissue damage after radiation for various types of cancer have been published.23–28 None of the genetic variants identified in this study emerged as risk factors in these prior investigations.

This is the first radiogenomics study in the field of ocular oncology suggesting that genetic factors may influence normal tissue response to ionizing radiation in patients with choroidal melanoma. However, this study was limited by the small sample size and the relatively short follow-up time chosen to identify patients with vision loss from radiation complications. Because manifestation of radiation vasculopathy is quite delayed in onset from the time of exposure, selection of time point beyond 3 years after treatment may have more accurately identified patients with the radiosensitive phenotype. This may have enhanced our ability to detect genetic variation associated with response to radiation injury. However, this approach would have resulted in an even smaller sample size. Further studies with larger cohorts and longer follow-up are needed to more definitively identify possible genetic influences in determining risk of vision loss after ocular irradiation for choroidal melanoma.

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