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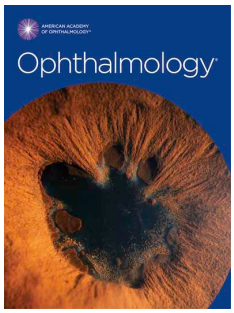

Genetic Profiling of Primary Orbital Melanoma-An Analysis of 6 Cases with Clinico-Pathological Correlation

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Running Title: Genetic profiling of primary orbital melanoma

Introduction

Primary orbital melanoma accounts for less than 1% of all primary orbital neoplasms.[1] In the largest clinico-pathological series on this subject to date, all 21 cases occurred in Caucasian patients, with a mean age at diagnosis of 42 years. Of these cases, 19 (90%) were associated with an orbital blue naevus. Of these 19 cases; 10 cases also showed some form of congenital melanosis (naevus of Ota or ocular melanocytosis).[2] Death from metastatic tumour occurred in 38% of cases, after a mean of 4.5 years follow up, with liver (88%) and brain (12%) being main targets of metastases.[2] A recent clinical study of 13 cases showed mortality from the disease in 5/13 cases with a mean survival of 44 months.[3] We present our experience of the clinical, histological and genetic profile from 6 cases of primary orbital melanoma and compare this with what is already known about uveal, cutaneous, and conjunctival melanomas.

Methods

This was a retrospective study performed on archival paraffin tissue surplus to diagnosis, held in the Histopathology Department, Royal Hallamshire Hospital Sheffield. All patients underwent standard written consent for the exenteration and incisional biopsy surgical procedures. Institutional Review Board/ Ethics Committee approval was obtained (The study was approved nationally (15/NW/0239) and by the Sheffield Teaching Hospitals Research & Development Office, under study number STH 19478, sub-study to STH 15427) for the use of anonymised retrospective formalin-fixed paraffin tissue, according to the UK Human Tissue Act (HTA) guidelines that governs the research use of such material that is surplus to

diagnosis. The research adhered to the tenets of the Declaration of Helsinki. The study was funded by the Sheffield Ocular Oncology Fund.

The clinical presentation / course and radiological features of patients were obtained from clinical records held in the Medical Records Department and from the Radiology Departments of the Royal Hallamshire Hospital Sheffield UK respectively. All histopathology data was obtained from slides and results held in the National Specialist Ophthalmic Pathology Service (NSOPS) archive, in the Department of Histopathology at the same hospital.

Inclusion Criteria for study

The inclusion criteria for the study were the presence of a primary orbital melanoma, with no clinical /radiological/imaging or other investigative modality evidence of intraocular, conjunctival, skin (including eyelid), mucosal (non-conjunctival) melanoma.

Tissue fixation and Immunohistochemistry

All surgically sampled tissue was fixed in standard 10% buffered formalin and exposed to standard processing to paraffin wax. 4 micron sections were cut and stained with haematoxylin and eosin (H&E). All cases were exposed to BAP-1, Melan A, HMB45 and Sox-10 immunohistochemistry. BAP1 retrieval of antigen was with pH8 (high pH) Dako retrieval solution. BAP1 antibody (Santa Cruz, California, Clone–C4; SC28383) was used at a dilution of 1:400 for 50 minutes, followed by a mouse link amplification step for 10 minutes, the Dako Flex Envision system HRP step for 20 minutes and finally DAB for 5 minutes. Melan A-Retrieval of antigen was with Agilent High pH EnV FLEX target retrieval solution. Melan A antibody (Agilent USA Clone A103) was used as a ready to use solution for 20 minutes, followed by

Agilent EnV FLEX HRP for 20 minutes and DAB for 5 minutes. HMB45- Retrieval of antigen was with Agilent High pH EnV FLEX target retrieval solution. HMB45 antibody (Agilent USA, Clone HMB45) was used as a ready to use solution for 20 minutes, followed by mouse link amplification step for 10 minutes and then Agilent EnV FLEX HRP for 20 minutes and DAB for 5 minutes. Sox10- Heat induced epitope retrieval was performed using Leica Bond Epitope Retrieval Solution for 2 minutes at 99^{o}C (high pH, Leica, AR9640). Peroxide block was applied for 5 minutes (as per detection kit) followed by application of SOX10 (CellMarque rabbit monoclonal EP268, diluted 1/200, cat. no. 385R-15) for 15 minutes. The Leica Bond III immunostaining platform was used, with Leica Bond Polymer Refine Detection with a DAB chromogen (Leica, DS9800).

DNA extraction, array comparative genomic hybridisation (array CGH), PCR and Sanger sequencing

DNA from 6 cases of primary orbital melanomas was extracted from formalin-fixed paraffin-embedded tumour material as previously described.[4] Array comparative genomic hybridization (aCGH) was performed on all 6 cases as detailed previously.[4] Sequencing for mutations of *GNAQ*, *GNA11* and *BRAF* was performed as detailed previously.[5, 6] Amplification of *NRAS*, *SF3B1* and *EIF1AX* regions was performed by standard PCR. PCR reagent concentrations were 1.5 mM $MgCl_2$, 10 pmol/µl primers and 12.5 mM dNTPs. [7-9] Due to the TERT promoter region being G-C rich, the protocol was adapted using a GC rich PCR system (Roche, Basel, Switzerland).[10] PCR conditions were 0.5 µM $MgCl_2$, 30 pmol/µl primers and 12.5 mM dNTPs. PCR product size was verified by agarose gel electrophoresis. Table 1 summarises the primers used. Following amplification, PCR products were purified to remove PCR

reagents using a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). Sequencing reactions were performed using a BigDye Terminator V.3.0 Cycle Sequencing Ready Reaction Kit (Life Technologies, Carlsbad, USA). Sequencing traces were analysed using FinchTV software (Geospisa Seattle, USA).

Results

Clinical and Radiological findings

Table 2 summarises the clinical and radiological features of the 6 cases. All patients were Caucasian and comprised 4 males (age range 65 to 91 years) and 2 females (26 and 65 years), with a male to female ratio of 3:1. The mean age at diagnosis was 66 years (range 26-91 years). The mean follow-up after histological diagnosis was 39 months (range 6 weeks to 84 months). Proptosis was common at presentation, and one case (case 1) showed episcleral and scleral pigmentation, without eyelid skin changes, indicative of ocular melanocytosis. None of the cases had clinical evidence of conjunctival, uveal, eyelid skin or systemic melanoma. Radiologically, what was particularly striking was the proximity of the melanomas to extraocular muscles, either located adjacent to the insertion or the body of the muscles or focally invading the muscle. No cases showed extension of the orbital mass beyond the bony orbit. Case 4 showed concurrent metastatic disease in the liver and bones.

Histopathology findings (see Figure 1)

These are summarised in Table 3. Most tumours comprised a variable mixture of spindle and epithelioid melanoma cells that were positive for melanocytic markers Melan A, HMB45 and Sox10. 2/6 cases had balloon cell change. 1/6 cases showed histological confirmation of ocular melanocytosis (case 1) and in a further 2 cases, benign spindle melanocytes were present around and beyond the orbital melanomas

(case 2 and 3). Balloon cell changes were seen in cases 4 and 6 but not in the other cases.

Array CGH for Chromosomal copy number changes (See Figure 2)

Array CGH data was analysed using Agilent Genomic Workbench Software v.6.0 (Agilent Technologies) and Nexus Copy Number Software v8.0 (BiodiscoveryH). Findings using both software's were comparable and revealed targeting of individual chromosomes rather than widespread genomic imbalance. The results for each tumour are presented in Table 4. The chromosomal copy number changes are summarised in Fig 2. The most frequent gains were of 6p (5/6), 8q (4/6), 17q (4/6), 6q (2/6), and 20p (2/6). The most frequently lost regions were 1p (2/6), 9p (2/6), 16q (2/6), 17p (2/6).

Mutational Analysis

Mutational profiling of genes commonly mutated in melanoma was performed using standard PCR and Sanger sequencing. The genes and mutational hotspots analysed are described in Table 4. Based on mutational data there is a suggestion of 2 distinct subgroups emerging in orbital melanomas. Those that exhibit mutations in *GNAQ, GNA11* or *SF3B1* (cases 1, 2 and 3) and those that contain mutations in *TERTp* and *NRAS* (cases 5 and 6). Case 4 did not exhibit mutations in any of the sites analysed, however it is worth noting that data for *EIF1AX* and *TERTp* mutations was not available due to poor quality DNA extracted from this sample. Cases 1 and 3 contained different missense substitutions at codon 625 of *SF3B1* (case 1 exhibiting a missense substitution of C>G and case 3 exhibiting a C>T substitution). Both mutations of *SF3B1* (R625G and R625C) have previously been reported to be

present in primary UM. [11-13] Case 1 also exhibited an R183Q mutation in exon 4 of *GNAQ.* This is an interesting observation as a mutation at this site is much rarer compared to the Q209 site (2.8% versus 44.8% in primary UM). [6] Case 2 exhibited a Q209L missense substitution of A>T at codon 209 of GNA11, a mutation seen in approximately 40-50% of UM cases.[6, 14-16] Cases 5 and 6 both exhibited mutations in the genes *NRAS* and *TERTp* (table 3). Both cases harboured a G12V missense substitution of G>T in codon 12 of *NRAS* and a C250T missense substitution of C>T in the promoter region of *TERT*.

Discussion

This study has documented the clinical, histological and molecular genetic findings for 6 cases of primary orbital melanoma. The clinical and histological findings concur with a previous study by Tellado et al [2], who documented 21 cases of primary orbital melanoma, which showed that the histology of orbital melanoma was very similar to UM. The melanoma cell types presented here comprised a variable mix of spindle and epithelioid cells and in some cases, extracellular matrix networks seen, as in UM. The primary orbital melanomas had a striking tendency to occur next to or within extraocular muscles. Most cases of primary orbital melanoma are thought to arise from orbital benign melanocytes or blue nevi, within or without the setting of oculo(dermal) melanocytosis.[2] These benign melanocytes tend to distribute along orbital fascial planes or within extraocular muscle, which would explain why in 5/6 cases, the melanomas were located as they were.

Case 1 featured ocular melanocytosis and showed a genetic profile identical to UM (monosomy 3 (M3) and gain of 8q), with loss of BAP1 protein nuclear expression

and featured liver metastases. As Changes of M3, 8q+ and loss of BAP1 protein nuclear expression, have all been significantly correlated with the development of hepatic metastases in UM, the observation of liver metastases in case 1 is perhaps not unduly surprising. This could represent a misclassified case of UM with secondary spread to the orbit from the ipsilateral or contralateral uvea. However, the benefit of exenteration histological examination showed no evidence of active or regressed lesions of UM in the uvea making it highly unlikely that it represented a UM. Similarly, none of the remaining exenterated cases showed evidence of uveal or conjunctival pathology, confirming that the orbital melanomas were indeed primary tumours. Interestingly, case 1 also showed a mutation in *SF3B1,* which, in the context of UM, is rarely reported in conjunction with loss of BAP1 expression. [11] Case 1 also contained a rare mutation of *GNAQ,* not often observed in UM. [6]

There is a wealth of data on the genetic alterations of UM [17-21], with less known about conjunctival melanomas [22-24], and there are no reports about the mutational and global chromosomal analysis of primary orbital melanomas. The findings of this investigation confirm that primary orbital melanomas share similarities with other forms of melanoma. The most common change (6p+), found in 5/6 primary orbital melanomas, is consistently reported for cutaneous, UM (including iris) and conjunctival melanoma.[21, 24-29] Other alterations, although less frequent (1p- and 8q+), are also reported across the spectrum of melanoma.[22] In contrast M3 found in one case is characteristic of UM, whilst other changes such as 17q+ are rarely observed in UM but have been reported for conjunctival melanoma.[20-22, 25, 26] Likewise, mutations of *TERTp* occur in conjunctiva melanoma but not UM, and *GNA11* and *SF3B1* are associated with UM but not conjunctival melanoma.[6, 13, 22, 23] When the cases are separated on the basis of mutational profile in combination with

genetic imbalances, it is apparent that cases 1, 2 and 3 are more akin to UM (M3 and 8q+ with *GNAQ, GNA11* and *SF3B1* mutations), whilst cases 4, 5 and 6 have mutations of *NRAS 12* and *TERTp* and chromosomal imbalances similar to those of conjunctival melanoma. Iris melanomas equally have been reported to have a mixed genotype, sharing mutations of both cutaneous (*BRAF/ NRAS*) and posterior UM (*GNAQ/ GNA11* and *SF3B1*), [29] but the segregation on the basis of mutations is not as distinct as seen here for the orbital melanomas. It is also remarkable that two of the cases (Case 5 and 6) have very distinctive profiles, both having 16q-, evidence of i(17q) and a tight focal amplification of 20p, findings which, although similar to conjunctival melanoma [22], may suggest that primary orbital melanomas have their own characteristic changes. To exclude cross contamination, the analysis was repeated and confirmed the similarity of the genetic changes in these two cases.

It is important to also consider the locality of these primary orbital melanomas. Posterior UM, in particular those affecting the ciliary body, are more likely to have M3.[17-20] In this study Case 1, with both M3 and loss of BAP1 nuclear expression, was located in the posterior orbit towards the apex. In contrast cases 5 and 6 had relatively anterior locations compared to the other cases, and both had the anterior aspect of the tumour biopsied which abutted the conjunctiva. For these orbital melanomas, the mutational signature of *NRAS* and *TERTp* is shared with conjunctival and skin melanoma.[22, 23] It is tempting to speculate whether proximity of the primary orbital melanoma to the conjunctiva, or anterior orbit, imparts a conjunctiva-type genetic signature, possibly mediated via light exposure; compared to the posteriorly located orbital melanomas, which would be relatively unexposed to light and more UM-like in their genetic profile. This possibility could only be tested by mapping different parts of a primary orbital melanoma to assess whether it was

made up of a mixture of conjunctival melanoma-like and UM-like genetic signatures. On this point however, it is worth noting that all of the 3 cases with a more UM-like genetic signature (cases 1, 2 and 3) showed a benign precursor lesion whereas the other 3 cases did not; although 2 of these latter cases were biopsies which did not sample the background non-tumour tissue. In the remaining case, the melanoma did not show a benign background precursor. This may indicate a genuine absence of a precursor or effacement of a precursor lesion by the melanoma. Ocular melanocytosis is a risk factor for UM but not conjunctival melanoma.[30] Although speculative, the presence of a benign precursor lesion may be a surrogate marker of one of the two genetic subgroups for primary orbital melanoma suggested by the study.

Genetic changes are powerful prognostic biomarkers for UM, but far less so for conjunctival melanoma. Poor prognosis for UM can be assigned by the presence of M3, 8q+ and 1p-, whilst 6p+ is usually associated with a better outcome and mutations of *SF3B1* and BAP1 loss also associate with metastasis.[15, 17, 18, 20] Given these associations Case 1 has all the classic features of a poor prognosis UM (1p-, M3 8q+, and absent BAP1 nuclear staining), and it is not perhaps surprising under these circumstances that the patient died from associated hepatic metastases. The other 2 cases with a more UM-like profile (2 and 3), had no metastases at the point of study, but did have some characteristic indicators of poor prognosis; including those that may predispose to metastasis over a longer period.[7, 13, 20, 25, 31, 32] Extended observation may clarify the association. For cases 4, 5 and 6, there was no consistent biomarker that related to the development of metastasis, and just as with conjunctival melanoma, further biomarkers would be advantageous. A recent study found mutations present in the *SF3B1* gene in 4/12 orbital melanomas and

suggests these mutations are associated with a better outcome in this tumour type. However, this study was limited to analysis of chromosomes 1, 3, 6 and 8 and therefore correlations to a non-UM profile could not be made from this series. [33]

In summary, we have presented the genetic profiles of 6 cases of primary orbital melanoma, which suggests that there may be two potential genetic groups, one of which may associate with melanocytosis / benign precursors. However, the study is limited by the analysis of 6 cases. Studying a larger cohort of cases will hopefully allow a prognostic stratification based on clinical, histological and molecular features, similar to current prognostic strategies for UM.[34] Secondly, patients with ocular melanocytosis who develop proptosis should be imaged urgently to rule out primary orbital melanoma.

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Figure 1 Histology images and immunohistochemistry findings.

A-Haematoxylin and Eosin (H&E) stained section showing a spindle cell rich area of primary orbital melanoma (Case 1).

B- An epithelioid cell rich area (Case 2).

C-Focal clear cell changes seen in cases 4 and 6.

D-The melanoma (bottom) abutting extraocular muscle (top).

E- Sox 10 nuclear positivity of primary orbital melanoma.

F-Background benign pigmented melanocytes present in background orbital soft tissue around and beyond some cases of primary orbital melanoma (Case 2).

G-Case 1: immunohistochemistry with BAP1, showing absent nuclear staining and some staining of the cytoplasm (Case 1).

H-Case 2: immunohistochemistry with BAP1, showing nuclear staining (Cases 2 to 6 showed this pattern of staining).

Figure 2 Array CGH profiles form 6 orbital melanomas, segregated on the basis of mutational signatures and copy number aberrations.

The cases were broadly divided into those melanomas that had mutations common to UM and those with mutations more frequent amongst conjunctival and cutaneous melanoma. Cases 1, 2 and 3, either had a *GNAQ*, *GNA11* or a *SF3B1* mutation and / or chromosome alterations commonly associated with UM such as M3 and 8q+ (often specifically in the form of an i(8q) as likely in case 3). Cases 4, 5 and 6, had mutations reported for conjunctival melanomas and chromosome changes less frequent in UM, but sometime reported for conjunctival melanoma.

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Gene	Exon	Forward Primer Sequence 5′-3′	Reverse Primer Sequence 5′-3′	Reference
GNAQ	5	AGAAGTAAGTTCACTCCATTCCC	TTCCCTAAGTTTGTAAGTAGTGC	5
GNAQ	4	TCTTTTTCTCCCACCCCTTGC	TTGTTTTGAAGCCTACACATGATTCC	6
GNA11	5	CGCTGTGTCCTTTCAGGATG	CCTCGTTGTCCGACT	5
GNA11	4	GTGCTGTGTCCCTGTCCTG	GGCAAATGAGCCTCTCAGTG	6
BRAF	15	TCATAATGCTTGCTCTGATAGGA	GGCCAAAAATTTAATCAG	5
NRAS	2	CGGTGTTTTTGCGTTCTCTAGTC	TCCGACAAGTGAGAGACAGGAT	9
NRAS	3	TTGAGGGACAAACCAGATAGGC	CCTTCGCCTGTCCTCATGTATT	9
SF3B1	15	TGATTATGGAAAGAAATGGTTGAAG	CATGTTCAATGATTTCAACTAAACTTC	8
EIF1AX	1	GAAAAGCGACGCAAAGAGTC	CTGGGTGACCTGCAATCTAC	8
TERT	promoter	GTCCTGCCCCTTCACCTT	GCTTCCCACGTGCGCA	10

Table 1. Primer sequences used in study

Case	Sex	Laterality	Presentation	Radiology	Post biopsy treatment	Post surgical treatment	Clinical course
1	M	L	Reduced VA and pain 2/52; 4 mm proptosis and slight upward globe displacement. RAPD	MRI: Posterior 22mm MD left fusiform mass abutting medial rectus with compression of optic nerve. Body PET-clear.	SSOE	Post-op orbital radiotherapy	No local recurrence. Miliary type liver metastases and epigastric lymphadenopathy 24 months after orbit surgery. Died 36 months after orbital diagnosis
2	M	R	Puffiness around R eye; inferotemporal 6mm proptosis	MRI-Equatorial 44mm MD supero-nasal mass above superior and medial rectus. No extrorbital spread. Body PET-clear	SSOE	Post-op orbital radiotherapy	No local recurrence and no metastases to date. Well and alive.60 months post-surgery
3	F	L	Left proptosis and left sub conjunctival haemorrhage VA 6/6 ; left 6th nerve palsy	MRI: Posterior 26mm MD well-defined mass around lateral rectus and adjacent to lacrimal gland. Body PET-all clear.	SSOE	Post-op orbital radiotherapy	No local recurrence and no metastases to date. Well and alive 36 months post-surgery.
4	F	L	3/12 proptosis	CT-extensive homogeneous orbital mass and multiple liver and bone metastases	nil	No treatment. Systemic palliative support.	Died 8 weeks after orbital biopsy from multiple bone and liver metastases.
5	M	L	Painless loss of vision; RAPD, proptosis; restricted eye movements	CT and MRI- left fusiform mass abutting medial rectus mass. CT whole body-no masses	nil	No treatment. Systemic palliative support	Died 6 weeks after orbital biopsy from cerebral metastases.
6	M	R	Supero-temporal mass. Diplopia on R gaze	CT- Anterior 26mm MD supero-lateral ovoid mass overlying insertions of superior rectus, superior oblique and lateral rectus. Separate from lacrimal gland. CT whole body-all clear	SSOE	No local treatment	No local recurrence and no metastases. Died of unrelated causes 48 months post-surgery.

Table 2 Summary of clinical and radiological features of the 6 cases

M (male); L(left); R(right); VA (visual acuity); RAPD (relative afferent pupillary defect; MD (maximum dimension); SSOE (Skin sparing orbital exenteration)

Case number	histology	Melanocytosis?	BAP1 immunohistochemistry
case 1	Exenteration: Posterior melanoma invading EOM; Central Nec with melanophages; mostly Sp cells & some Ep cells. No LVS; No PN ; HMB45+ MelanA+ Sox10+. No conjunctival or uveal melanoma.	Yes-melanocytosis of choroid, sclera, episclera and orbit soft tissue.	Absent nuclear expression
case 2	Exenteration: Superior equatorial melanoma; Sp &E cells; packeted architecture; vascular invasion; No PN; Melan A+HMB45+ Sox10+. No conjunctival or uveal melanoma.	Yes-scattered benign spindle cells in orbit soft tissue around melanoma.	nuclear expression
case 3	Exenteration: Posterior orbital melanoma; Sp cells; Nec; No LVS; No PN; Melan A+, HMB45+ Sox10+; No conjunctival or uveal melanoma.	Yes-scattered benign spindle cells in orbit soft tissue adjacent and distant from melanoma	nuclear expression
case 4	Incisional biopsy (taken from anterior orbit): Melanoma; Sp &Ep cells with focal balloon cell change; packeted architecture. Melan A+, HMB45+, Sox10+	Not assessable histologically	nuclear expression
case 5	Incisional biopsy (taken from anterior medial orbit) : Melanoma; Sp &Ep cells Melan A, HMB45 Sox10+	Not assessable histologically	nuclear expression
case 6	Exenteration: Anterior orbital melanoma; Ep cell rich; balloon cell change; No LVS; No PN; Melan A+HMB45+; Sox10+; No conjunctival or uveal melanoma.	No	nuclear expression

Table 3 Summary of the histological findings.

Key: EOM-extraocular muscle; Nec-necrosis; Sp-spindle; Ep-epithelioid; LVS-lymphovascular space invasion; PN-perineural invasion;

case no.

	GNAQ	GNA11	SF3B1	EIF1AX	BRAF	NRAS	TERT	gain of chromosomal copy number	loss of chromosomal copy number
1								8q (partial)	3, 1p (partial)
2								6, 8q, 9, 10, 11, 13, 17, 21	19
3								1p (focal), 6p (partial), 17q(partial), 20q (focal)	1p (partial), 4q (partial), 8p (partial), 9p (partial)
4								6p (partial), 7p (focal), 8	none
5								1p (partial), 6, 13q (partial), 17q, 20p (focal)	16p (focal), 16q (partial), 17p, 20q (focal)
6								6p, 17q, 20p (focal)	9 (focal), 10, 16 (partial), 17p, 20q (focal), 21

* focal losses and gains not reported in table were identified as CNVs due to unmatched control DNA used for aCGH

** where wildtype reported for GNAQ, GNA11 and NRAS, indicates wildtype for all mutational sites analysed as outlined in table 1

SF3B1 (R625)

GNAQ (R183)

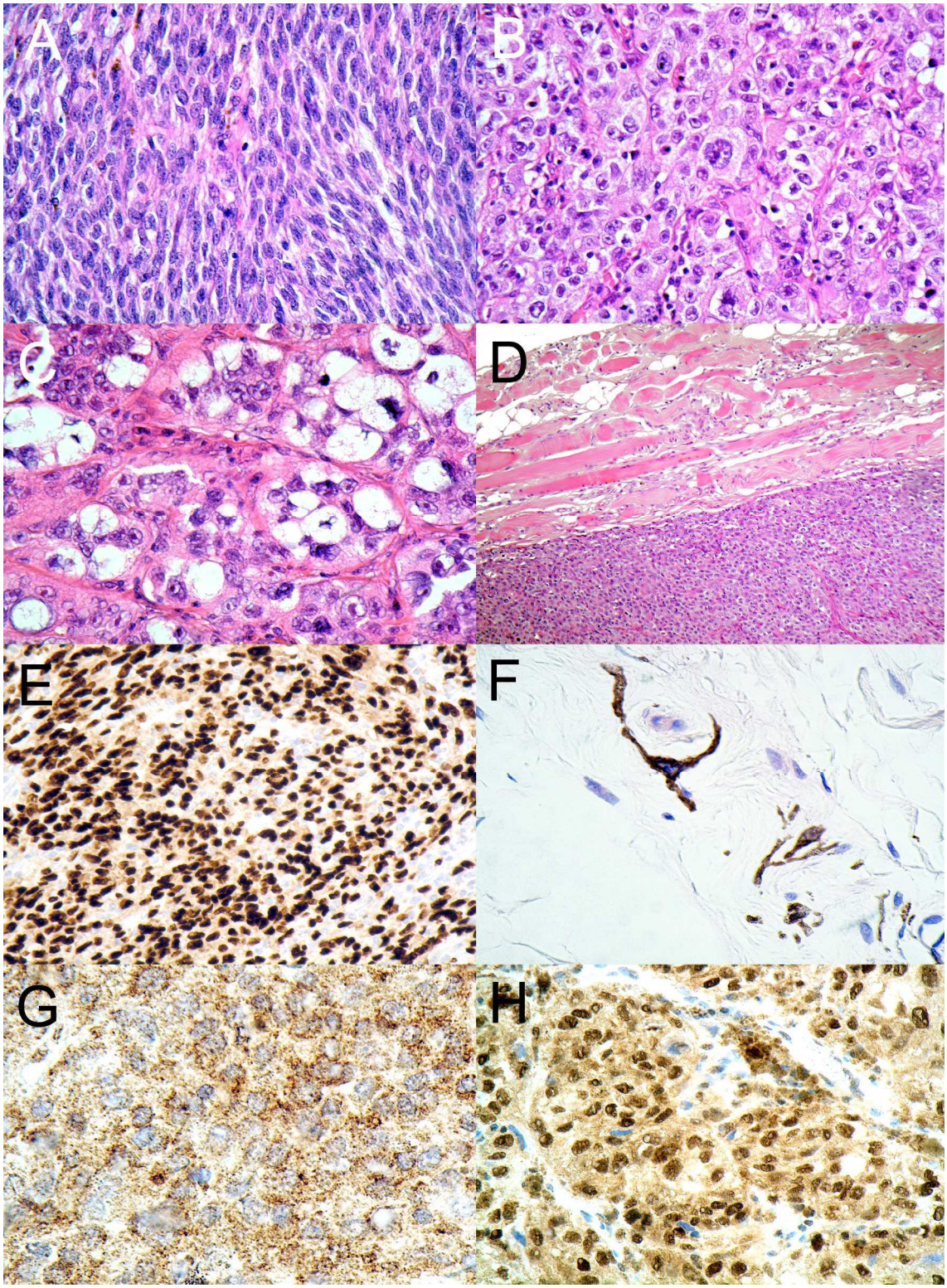
GNA11 (Q209)

NRAS (G12)

TERT (p.146)

wildtype

failed


A
B
C
D
E
F
G
H

Highlights

The study presents the genetic profiles of 6 primary orbital melanomas. The data suggests there are 2 subgroups: A uveal-like signature and a conjunctival-like signature, with the uveal-like group possibly associated with benign precursors.