**BRAF, NRAS, and GNAQ Mutations in Conjunctival Melanocytic Nevi**

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**Purpose.** To evaluate **BRAF**, **NRAS**, and **GNAQ** mutations in surgical specimens of common and blue conjunctival melanocytic nevi.

**Methods.** Surgical specimens from 25 conjunctival melanocytic nevi (23 common and 2 blue) of 25 patients were evaluated. All common nevi were analyzed immunohistochemically for the expression of **BRAF** V600E or **NRAS** Q61R. One lesion with negative immunoreactivity for both blue nevi, a hybridization capture-based next-generation sequencing method was employed for mutation analysis. For common nevi, genetic features were compared with clinical and histopathologic findings. Continuous variables (age at excision and largest basal diameter) were compared with a Students’s t-test and all categoric variables were compared with Fisher’s Exact Test.

**Results.** Of common melanocytic nevi, 9 (39.1%) were immunoreactive for **NRAS**Q61R and 13 (56.5%) were immunoreactive for **BRAF** V600E. One common nevus, which was immunonegative for both **BRAF** V600E and **NRAS** Q61R was found to harbor an **NRAS**Q61K mutation by sequence analysis. Patients with **NRAS**-mutated nevi were more likely to report occurrence of the lesion prior to 18-years old and more likely to have intrinsic cysts. The mean largest basal diameter was 6.0 and 3.5 mm for **NRAS**- and **BRAF**-immunoreactive lesions, respectively (P = 0.003). **GNAQ** mutations were identified in each of the two blue nevi of this study.

**Conclusions.** These findings document that common conjunctival melanocytic nevi have mutually exclusive mutations in **BRAF** and **NRAS**. The two conjunctival blue nevi harbored **GNAQ** mutations. This suggests the driver mutations of conjunctival nevi are similar to those of nevi of the skin. At the molecular level, conjunctival nevi appear more like cutaneous nevi than choroidal nevi.

Keywords: conjunctiva, nevi, genetics, melanoma, eye

There are many parallels between melanocytic lesions of the skin and those of the conjunctiva. In both tissue types the originating melanocytes are derived from the neural crest and migrate toward the epithelium (or extraepithelial in the case of blue nevi) in their respective locations. There has been some evidence that they share the similar genetic underpinnings, however, this has not been fully expounded upon in the literature. For instance, cutaneous nevi have mutually exclusive mutations in **NRAS** and **BRAF** and these are shown to associate with particular clinic-pathological features.1–4 Even though conjunctival melanomas (for some of which conjunctival nevi are thought to be precursor lesions) have mutually exclusive mutations in **NRAS** and **BRAF**,5–9 only **BRAF** mutations have so far been documented in conjunctival nevi. Furthermore, the majority of cutaneous blue nevi have a unique genetic identity with mutations in **GNAQ** or **GNA11**, and the presence of these mutations have been confirmed in oral and central nervous system blue nevi,10–15 but not in the conjunctiva.

Thus, the purpose of this study was to evaluate the mutation spectrum of conjunctival melanocytic nevi. With the availability of mutant protein-specific antibodies we focused on documenting the distribution of **BRAF** V600E and **NRAS** Q61R expression in common conjunctival melanocytic nevi and correlated the mutation status with clinicopathologic features. Using molecular studies we also document for the first time the presence of **GNAQ** mutations in blue nevi of the conjunctiva and the presence of an **NRAS**Q61K mutation in a common nevus.

**Methods.**

The institutional review board of Memorial Sloan Kettering Cancer Center and Emory University School of Medicine approved this retrospective study. It included 25 conjunctival nevi from 25 patients between September 2013 and May 2017. Lesions included in this study underwent consecutive surgical excision within the study timeframe and had histopathologic confirmation by a pathologist. Informed consent was obtained from each patient.
Patient data included sex, age, and ethnicity. Clinical data included initial occurrence of conjunctival nevus (juvenile [18 years or younger] or adulthood), age at time of excision, ocular site (bulbar, palpebral, caruncle), site of ultraviolet light exposure (intrapalpebral fissure or otherwise), iris pigmentation, largest basal diameter, degree of pigmentation (amelanotic, melanotic, deeply melanotic, or amelanotic/melanotic), thickness (flat or raised), and presence of intrinsic cysts, intrinsic vessels, or sentinel vessels. Location was defined histopathologically (compound, subepithelial, etc.). Clinical data was not available for the de-identified conjunctival blue nevi specimens. Representative clinical images are shown in Figures 1, 2, and 3.

Tissue sections, 5-μm thick were cut from formalin-fixed and paraffin-embedded tissue blocks. For detection of BRAFV600E, an automated immunohistochemical system (Ventana BenchMark XT; Ventana Medical Systems, Inc., Tucson, AZ, USA) was used with the commercially available mouse monoclonal antibody VE1 (anti-BRAFV600E; Ventana). For NRASQ61R detection the commercially available rabbit monoclonal antibody SP174 (Spring Bioscience, Pleasanton, CA, USA) was used with the Leica Bond detection system (Leica, Wetzlar, Germany). The staining result was recorded as either homogeneous immunoreactive for the respective marker or completely negative.

For the single sample that did not reveal a mutation by the above method and the blue nevi, the MSKCC IMPACT assay was used as previously described, on formalin-fixed paraffin embedded tissue.

For statistical analysis, continuous variables (age, age at excision, and largest basal diameter) were compared with a Student’s t-test and all categoric variables (clinicopathologic parameters) were compared with Fisher’s Exact Test. Analysis was performed with Prism 7 (Graphpad Software, Inc., La Jolla, CA, USA) and a P value < 0.05 was considered statistically significant.

RESULTS

Of nevi, nine (40.9%) were immunoreactive for NRAS Q61R and 13 (56.5%) were immunoreactive for BRAFV600E mutations. One specimen lacked immunoreactivity for all immunohistochemistry targets, for which the MSK-IMPACT assay identified an NRASQ61K mutation. Both blue conjunctival nevi had a mutation in GNAQ (Q209I and Q209H) detected by MSK-IMPACT.

For the common nevi, the median age at the time of excision was 13.5 and 29 years for patients with an NRAS and BRAF mutated lesion, respectively ($P = 0.09$). The Table shows associations of NRAS and BRAF expression or mutational status with clinicopathologic features. There were three parameters that showed a statistical difference between patients with an NRAS- and BRAF-immunoreactive lesion: patients with NRAS-immunoreactive lesions were significantly more likely to report occurrence of the lesion prior to 18-years old. In addition, NRAS-immunoreactive lesions were significantly more likely to
have intrinsic cysts and have a largest basal diameter more than 5 mm. The mean largest basal diameter was 6.0 and 3.5 mm for NRAS- and BRAF-immunoreactive lesions, respectively ($P = 0.003$).

At a mean follow-up of 13.1 months, there were not events of recurrence or malignant transformation.

**DISCUSSION**

Ocular nevi differ in their genetic underpinnings depending on the anatomical derivation of their melanocytes. Nevi derived from uveal tract melanocytes harbor GNAQ/11 mutations.17 On the contrary, conjunctival nevi are more akin to their cutaneous counterpart and have been shown to have BRAF mutations.7 The present study confirms the similarity between cutaneous and conjunctival nevi by identifying further genetic aberrations and associations with clinical features that are found in cutaneous nevi.

Cutaneous nevi have NRAS and BRAF mutations.1-2 BRAF is a serine-threonine kinase and NRAS an isoform of the RAS family of GTPase proteins; and both activate the MAPK signaling cascade, which leads cell cycle progression and cell proliferation. Similarly, for all the conjunctival nevi in this study, immunoreactivity and mutational analysis allowed for identification of either an NRAS or BRAF aberration. For this cohort, NRAS and BRAF mutations occurred in a mutually exclusive manner, with just over half the lesions exhibiting immunoreactivity with the antibody VE1, thereby indicating the presence of a BRAFV600E mutation.

For cutaneous nevi, the presence of either a BRAF or NRAS mutation has been associated with specific clinical features.1-4 For instance, congenital cutaneous nevi are more likely to harbor NRAS mutations, while acquired nevi have a propensity toward BRAF mutations.1-3 One prior study on conjunctival nevi, that evaluated for BRAF mutations only, found no difference in the proportion of children and adults with mutant lesions.7 On the contrary, in our series, NRAS mutations were statistically more frequent in patients reporting the occurrence of their conjunctival nevus prior to the age of 18 years. However, the retrospective nature of this study limited our understanding of each lesion's occurrence to patient reporting. Furthermore, conjunctival nevi, particularly those that are congenital, are commonly amelanotic and difficult to fully appreciate overlying the white sclera; thereby, possibly influencing the recognition of their true occurrence date.

In cutaneous nevi, there is evidence to suggest that larger congenital lesions more commonly harbor NRAS mutations, while smaller lesions may have either BRAF or NRAS mutations.2 In line with this finding, in the present study, conjunctival nevi with a basal diameter greater than 5 mm

![Image](http://arvojournals.org/)
were statistically more likely to have NRAS-immunoreactivity 
\( (P = 0.006) \). Evaluated another way, the mean largest basal 
diameter was significantly greater (almost double) in NRAS-
immunoreactive lesions compared with BRAF-immunoreactive 
nevi. Finally, intrinsic cysts were statistically more likely in 
NRAS-immunoreactive lesions; which may be a clinical marker 
for juvenile/congenital nature of these lesions.

The risk of malignant transformation of conjunctival nevi 
into melanoma is predicted to be 1% (Gerner et al.\textsuperscript{18}), 
and more common in adult-onset lesions compared with those 
that appear in childhood. Like conjunctival nevi, up to 14% to 
50% of conjunctival melanomas have been found to harbor 
\textit{BRAF} mutations and 18% have \textit{NRAS} mutations (Griewank et 
\al.,\textsuperscript{5} Lake et al.,\textsuperscript{6} Goldenberg-Cohen et al.,\textsuperscript{7} Gear et al.,\textsuperscript{9} 
Riechardt et al.\textsuperscript{5}). In one study, \textit{BRAF} mutant conjunctival 
melanomas were associated with a caruncle location and in 
another study with young age, male sex, sun-exposed 
location, mixed/nonpigmented color and nevus origin (Lars-
en et al.,\textsuperscript{19} Griewank et al.\textsuperscript{3}). However, this latter study found 
no association between \textit{BRAF} mutant status and prognosis. 
Along the same lines, Gear and colleagues\textsuperscript{5} found no 
correlation between \textit{BRAF} mutations and location or other 
clinicopathologic characteristics (Gear et al.\textsuperscript{9}). Therefore, 
the implications of \textit{BRAF} mutations in conjunctival melanoma are 
controversial, and the potential of malignant transformation of 
nevi has an unknown association with \textit{BRAF} or \textit{NRAS} 
status.

It is proposed that lesions with \textit{GNAQ} mutations possess the 
unique traits of being derived from extraepithelial melanocytes 
and particularly those that originate from cranial neural crest 
cells.\textsuperscript{11} Therefore, it would be conceivable that 
conjunctival blue nevi would be \textit{GNAQ} mutant. This theory is 
strengthened by the discovery that cutaneous, central nervous 
system, and oral cavity blue nevi have \textit{GNAQ} mutations.\textsuperscript{10–15} In keeping 
with this prediction, the present study demonstrates \textit{GNAQ} 
mutations in both conjunctival blue nevi. Ultraviolet light 
exposure is not thought to play a role in \textit{GNAQ} mutations, 
and remains debatable in \textit{BRAF} and \textit{NRAS} mutations. This shared 
genetic aberration in both cutaneous and conjunctival derived 
nevi is another example of the similarity between these two 
lesions from differing (skin and conjunctiva) anatomic origins.

Immunohistochemistry techniques for \textit{BRAF V600E} and 
\textit{NRAS Q61R}, are highly sensitive and specific.\textsuperscript{20–22} In our 
series, there was only one patient with negative immunohis-
tochemistry for all targets. Instead, this specimen was 
evaluated with the MSK-IMPACT assay,\textsuperscript{16} which revealed a less 
common mutation in \textit{NRAS Q61K}, which is not recognized by 
the antibody SP174. The MSK-IMPACT assay is an invaluable 
tool with the advantage providing a combination approach for 
the detection of multiple categories of genetic alterations, and 
is particularly useful at identifying less common alterations that 
may not be easily detected through immunohistochemistry.

In summary, our findings confirm the parallels that exist 
between cutaneous and conjunctival melanocytic lesions. 
Our common conjunctival nevus cohort revealed data to support 
mutually exclusive genetic alterations of \textit{BRAF/V600E} and 
\textit{NRAS}, with slightly higher presence of the former. Like 
cutaneous nevi, the NRAS-immunoreactivity was more com-
mon in larger lesions with an earlier occurrence in life. The 
similarities extend to blue nevi where we demonstrate a 
common genetic aberration in \textit{GNAQ}, which were present in 
two blue nevi specimens. These findings would benefit from 
validation with a larger cohort study. Prior concerns of 
specialists in the field point to the semantic problems with 
classifying pigmented lesions of the conjunctiva.\textsuperscript{23} The 
association between genetics and histopathology of nevi was 
limited to blue versus nonblue nevi (and did not extend to 
distinguishing among common nevi); however, genetics did 
suggest an association with some clinical features of common 
nevi. These findings open up the possibility of organizing 
pigmented lesions molecularly or genetically, and this in turn 
may relate to pathogenesis and ultimately inform treatment 
approaches.

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