BRAF, NRAS, KIT, TERT, GNAQ/GNA11 Mutation Profile and Histomorphological Analysis of Anorectal Melanomas: A Clinicopathologic Study

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ABSTRACT

Objective: Primary anorectal melanomas (AMs) are uncommon neoplasms with aggressive behavior. Molecular profile and clinicopathologic features of AMs are still not well established. In this study, we aimed to investigate BRAF, NRAS, KIT, TERT, and GNAQ/GNA11 mutation status and clinicopathologic features of AMs.

Material and Method: All diagnostic slides of 15 AMs were reviewed. Histopathological and follow-up information were documented. Mutations in exon 15 of the BRAF gene; exons 2 and 3 of the NRAS gene; exons 9, 11, 13, 17, and 18 of the KIT gene; and exons 4 and 5 of the GNAQ/GNA11 genes and mutations in the promoter region of the TERT gene (chr.5, 1,295,228C>T and 1,295,250C>T) were analyzed.

Results: BRAF(V600E) and KIT(V555I and K642E) mutations were observed in one (7%) and two cases (14%), respectively. NRAS, TERT and GNAQ/GNA11 mutations were not detected. The mean age was 65. Patients presented with rectal mass, rectal bleeding, pain, and weight loss. 73% of the lesions were macroscopically polypoid. The most common tumor cell type was epithelioid. Mean tumor thickness was 10.4 mm. One third of the cases lacked pigmentation. In situ melanoma was present in one third of the cases. Among 14 patients with follow-up data, 12 succumbed to disease. The mean overall survival was 36 months.

Conclusion: AMs are uncommon tumors with dismal survival, usually occurring in the elderly in various gross and microscopic appearances. In terms of molecular profile, BRAF and KIT mutations are rarely detected. Profiling of larger cohorts is required to elucidate the pathogenesis and to identify potential molecular indicators that may contribute to the development of individualized targeted therapies.

Keywords: Anorectal melanoma, BRAF, NRAS, KIT, TERT, GNA

INTRODUCTION

Primary anorectal melanomas (AMs) are uncommon neoplasms that account for about 1% of anal canal tumors (1). Among mucosal melanomas, which constitute around 1% of all malignant melanomas (2), the anal canal is the second most common site of origin, following the head and neck (3). AMs are believed to arise from the melanocytes of the anal squamous epithelium and extend towards the anal canal (4,5); however, cases that originated from the rectal mucosa -without the involvement of the squamous epithelium- have also been reported (6,7).

Patients with AM usually present with rectal bleeding and pain. Tumors often mimic hemorrhoids, anal polyps or rectal carcinoma, forming large, dark-colored masses with expansile and nodular borders, with or without ulceration (8,9). Microscopically, tumors are often composed of sheets/fascicles of epithelioid or spindled malignant cells with vesicular chromatin and prominent nucleoli, with variable amounts of pigmentation (10). However, unusual presentations and rare histologic/cytologic patterns often challenge pathologists in the differential diagnosis of AMs, which includes carcinomas, sarcomas and even lymphomas (11,12). In challenging cases, a panel of immunohistochemical stains, including markers of melanocytic lineage is required to render the accurate diagnosis (13).

Similar to mucosal melanomas of other sites, AMs behave much worse than their cutaneous counterparts. Despite the use of various treatment regimens including extensive surgery, radiotherapy, chemotherapy, and targeted therapies, AMs have an aggressive clinical course with an overall
5-year survival rate of less than 25% (3,14). Additionally, AMs were associated with the poorest prognosis among mucosal melanomas in a large European cohort (15).

The recent progress in the molecular profiling of cutaneous melanomas has greatly contributed in our understanding of their pathogenesis, as well as their management with the use of targeted therapies and immunotherapy (16). However, mucosal melanomas tend to differ from their cutaneous counterparts in terms of molecular profiling; albeit showing a heterogeneous molecular profile, they have lower BRAF and TERT, and relatively higher NRAS and KIT mutation frequencies (17-27). Additionally, GNAQ/GNA11 mutations, which have been reported in uveal melanomas and subjected to targeted therapies (28,29), also occur rarely in AMs (30), but not in other mucosal melanomas (31). However, molecular profiles of mucosal melanomas are still not well established due to their rarity. In addition to their molecular background, data concerning their clinicopathologic features are highly limited. Accordingly, widely accepted treatment protocols do not exist. In this study, as an extension to our previous work on head and neck mucosal melanomas (31), we aimed to investigate the BRAF, NRAS, KIT, TERT and GNAQ/GNA11 mutation status of 15 AMs, as well as their clinicopathologic features.

**MATERIAL and METHODS**

**Case Selection, Clinical and Pathological Data Collection**

The digital database of the pathology department (Istanbul Faculty of Medicine, Istanbul University, Istanbul, Turkey) was searched for cases diagnosed as AM between the years 2000 and 2019, including both in-house material and outside consultations. Data on clinical history and physical/radiologic examination were reviewed for all retrieved cases in order to exclude previous history of cutaneous melanoma and/or the possibility of metastasis. Cases with a suspicion of secondary melanoma were not included. Diagnostic slides and paraffin blocks were retrieved from the archives. All slides (hematoxylin and eosin and/or immunohistochemically stained) were reviewed. Tumor cell types were classified under four categories (epithelioid, spindle, pleomorphic, and lymphoma-like), as mentioned in the literature (9,12,32,33), and combined morphology was assessed when appropriate. Histopathological information regarding tumor thickness (Breslow), presence of perineural and lymphovascular invasion, pigmentation, ulceration, necrosis, tumor infiltrating lymphocytes, mitotic count (per high power field), and margin status (when applicable) were also documented. Follow-up information was obtained from the clinical files or national database.

**Mutation Analysis**

Tumor targets (>90% viable tumor) were manually micro-dissected from 10-mm thick unstained histologic sections for enrichment of tumor cellularity. Deparaffinization of tissue sections was performed. Then, DNA was isolated by using the QIAamp DNA FFPE Tissue Kit (50) (catalog #: 56404) (QIAGEN, Hilden, Germany). DNA concentrations of the samples were assessed spectrophotometrically using a Nanodrop 1000 spectrophotometer (ThermoScientific, USA).

Mutations in exon 15 of BRAF gene; exons 2 and 3 of the NRAS gene; exons 9, 11, 13, 17, and 18 of the KIT gene; and exons 4 and 5 of the GNAQ and GNA11 genes (well-known hotspot regions for oncogenic mutations) and mutations in the promoter region of the TERT gene (chr5, 1,295,228C>T and 1,295,250C>T) were analyzed by validated previously described polymerase chain reaction (PCR)-based direct Sanger sequencing (analytical sensitivity 25%) by using 200 ng of each tumor DNA (31).

**Additional Information**

The Helsinki principles were respected in this study and patients’ data confidentiality was ensured according to their guidelines. This study was approved by the institutional review board.

**RESULTS**

**Clinical Features**

The study was conducted with 15 cases of 15 patients (8 males and 7 females) with a mean age of 65 years (range: 30 - 86 years). The specimens consisted of 8 local excisions, 3 abdominoperineal resections, 1 polypectomy and 3 incisional biopsies. By definition, all tumors originated from the anal canal. Among patients with available information (n=9), presenting symptoms were described as rectal mass, rectal bleeding, pain and weight loss.

**Histopathology**

Diagnostic slides of the fifteen cases were systematically reviewed. Tumors were polypoid in 73%. The cell type was epithelioid and spindle in 33%, epithelioid and lymphoma-like in 27%, spindle in 13%, epithelioid and pleomorphic in 13%, spindle and lymphoma-like in 7%, and lymphoma-like in 7% of cases (Figure 1). Mean tumor thickness in 12 cases was 10.4 mm (range: 1.1-22 mm). Tumor thickness could not be measured in 2 incisional biopsies and 1 polypectomy due to poor orientation. Majority of cases (67%) showed pigmentation, whereas 33% were amelanotic (Figure 2). Ulceration was seen in 80% of cases. Mean mitotic count was
Figure 1: Neoplastic cells in anorectal melanoma demonstrate various morphologic appearances: A) Epithelioid melanoma cells with roundish nuclei and wide eosinophilic cytoplasm, B) Lymphoma-like small neoplastic cells, admixed in a fibrous stroma, showing crush artifact, C) Pleomorphic melanoma cells with huge, bizarre nuclei and prominent cytoplasm, D) Spindle cells showing elongated nuclei and sparse cytoplasm (A-D: Hematoxylin&Eosin, x400).

Figure 2: A) Anorectal melanoma cells, which lack melanin pigment (Hematoxylin&Eosin, x200), B) Melanoma in situ, atypical melanocytes showing continuous growth at the basal layer (Hematoxylin&Eosin, x400).
4.9 per 10 high power fields (range 0-10). In situ melanoma was detected in 33% of the cases (Figure 2). Intratumoral lymphocytes were prominent in 53%. Metastasis in lymph nodes was observed in 3 abdominoperineal resections.

**Immunohistochemistry**

Among 15 cases, 10 were subjected to immunohistochemical analysis. Five cases that did not require immunohistological analysis harbored in situ melanoma component and/or prominent pigmentation.

Among melanocytic markers, S-100 and HMB-45 were positive in all cases (100%; n=9 and 7; respectively). Melan-A was positive in 6/7 (86%) cases. Epithelial markers (Pan-cytokeratin, epithelial membrane antigen, and carcinoembryonic antigen), neuroendocrine markers (chromogranin, synaptophysin), muscle markers (desmin, smooth muscle actin) were all negative when performed, along with leukocyte common antigen, CD30 and CD34. CD117 was positive in 2 of 3 cases performed.

**Mutation Analysis**

A total of 3 cases (20%) were found to harbor mutations. BRAF(V600E) and KIT(V555I and K642E) mutations were observed in one (7%) and two cases (14%), respectively. NRAS, TERT, and GNAQ/GNA11 mutations were not observed.

**Follow-Up and Survival Information**

Among 14 patients with available information, 12 died. The mean overall survival was 36 months (range: 0-112 months). The histopathological, clinical, and mutational findings and follow-up information are summarized in Table I.

**DISCUSSION**

Mutational profile of mucosal melanomas is known to differ from their cutaneous counterparts, suggesting a different pathway in the pathogenesis: They harbor lower BRAF and TERT, and relatively higher NRAS and KIT mutation frequencies (17-27). The absence of UV damage is often mentioned to be associated with this disparity. Furthermore, regarding the site of origin, differences also exist in the same subgroup: in an earlier study, we concluded that NRAS and TERT promoter mutation rates were significantly higher in sinonasal than in oral mucosal melanomas of the head and neck (31). In the current literature, data on AMs’ molecular profile is mostly merged with cutaneous and/or mucosal melanomas, primarily due to their rareness (34-39). In studies with relatively large cohorts of AMs, KIT mutations were most commonly encountered, followed by mutations in NRAS. The newly introduced NF1 gene also has an important role in the oncogenesis. BRAF mutations were also observed with different frequencies, most likely due to small sample sizes or populational differences of the cohorts (40-44). In addition, one study showed around 2% GNAQ and 6% GNA11 mutations (39). In our study group, BRAF and KIT mutations were found in 7% and 14%, respectively. NRAS, TERT, and GNAQ/GNA11 mutations were absent. Together, these supported the low mutation burden of AMs, as stated in the literature (45). In a large cohort of mucosal melanomas, 3% of AMs showed BRAF, 10% showed NRAS, and 19% showed KIT mutations. This study analyzed a subset of cases by Sanger sequencing, and others by next-generation sequencing (NGS); and proposed NRAS mutation as a predictor of worse survival, independent of stage in all mucosal melanomas (46). Those being mentioned, as a limitation of this study, we had a limited number of cases, impeding a correlation analysis between mutational status and prognostic data. We also did not have access to NGS techniques. Therefore we were unable to perform a comprehensive genomic analysis including NF1, which was recently integrated in the molecular classification of AMs.

Our findings verified that AMs are highly rare and aggressive neoplasms that generally occur in elder patients, with a mean age of 65 years in the present study. In one study, older age (>70 years) was found to be an independent poor prognostic factor (10). Although our data did not reveal any significant sex predilection, geographic and populational differences in the relative frequency between two genders have been reported (10,47).

Clinically, recognizing AMs can be challenging for physicians. The symptomatology may include non-specific rectal bleeding and pain, as well as weight loss in metastatic disease (48). Endoscopically, tumors can present with various appearances. Polypoid masses are frequently encountered, similar to 73% of our cases. Anal prolapse, and luminal or submucosal masses with or without ulceration or pigmentation can also be seen (48). This may cause misdiagnosis of AM as hemorrhoids, perianal abscess, anal polyps or other malignancies (49).

The presence of melanin pigmentation can help render the accurate diagnosis. However, it is not always present, with some studies reporting 37% of their cases as amelanotic (12,32). In addition, in situ melanoma component, or junctional melanocytic activity, which are characteristic in cutaneous melanomas, have been reported in up to 75% of AMs (12,32,49). However, this feature may be missing due to the absence of adjacent mucosa in incisional biopsies.
Table I: Clinicopathologic features of the study group.

<table>
<thead>
<tr>
<th>Case No</th>
<th>Age</th>
<th>Sex</th>
<th>Operation Type</th>
<th>Thickness (mm)</th>
<th>Ulceration</th>
<th>Mitosis (n/HPF)</th>
<th>In situ melanoma</th>
<th>Intratumoral lymphocytes</th>
<th>Vascular invasion</th>
<th>Perineural invasion</th>
<th>Necrosis</th>
<th>Pigment Cell type</th>
<th>Surgical margins</th>
<th>Patient status</th>
<th>Overall survival (months)</th>
<th>Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>82</td>
<td>M</td>
<td>Local excision</td>
<td>10.0</td>
<td>Present</td>
<td>3</td>
<td>Absent</td>
<td>Present</td>
<td>Absent</td>
<td>Absent</td>
<td>Present</td>
<td>Epithelioid &amp; spindle</td>
<td>N/A</td>
<td>Dead</td>
<td>70</td>
<td>BRAF et al (1799T&gt;A, V600E)</td>
</tr>
<tr>
<td>2</td>
<td>63</td>
<td>M</td>
<td>Local excision</td>
<td>22.0</td>
<td>Present</td>
<td>3</td>
<td>Present</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
<td>Present</td>
<td>Epithelioid &amp; pleomorphic</td>
<td>Positive</td>
<td>Dead</td>
<td>4</td>
<td>None</td>
</tr>
<tr>
<td>3</td>
<td>86</td>
<td>F</td>
<td>Incisional biopsy</td>
<td>N/A</td>
<td>Present</td>
<td>4</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
<td>Present</td>
<td>Epithelioid &amp; spindle</td>
<td>N/A</td>
<td>Dead</td>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td>4</td>
<td>68</td>
<td>M</td>
<td>Incisional biopsy</td>
<td>N/A</td>
<td>Present</td>
<td>0</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
<td>Epithelioid &amp; lymphoma-like</td>
<td>N/A</td>
<td>Lost to follow-up</td>
<td></td>
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</tr>
<tr>
<td>5</td>
<td>75</td>
<td>F</td>
<td>Local excision</td>
<td>9.4</td>
<td>Present</td>
<td>6</td>
<td>Present</td>
<td>Present</td>
<td>Present</td>
<td>Absent</td>
<td>Absent</td>
<td>Epithelioid &amp; pleomorphic</td>
<td>Positive</td>
<td>Dead</td>
<td>5</td>
<td>None</td>
</tr>
<tr>
<td>6</td>
<td>58</td>
<td>F</td>
<td>Local excision</td>
<td>7.7</td>
<td>Present</td>
<td>6</td>
<td>Present</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
<td>Epithelioid &amp; spindle</td>
<td>N/A</td>
<td>Alive</td>
<td>85</td>
<td>None</td>
</tr>
<tr>
<td>7</td>
<td>72</td>
<td>F</td>
<td>Local excision</td>
<td>11.0</td>
<td>Present</td>
<td>9</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
<td>Epithelioid &amp; lymphoma-like</td>
<td>Negative</td>
<td>Dead</td>
<td>28</td>
<td>KIT et al (1663G&gt;A, V555I)</td>
</tr>
<tr>
<td>8</td>
<td>71</td>
<td>F</td>
<td>Abdominoperineal resection</td>
<td>13.0</td>
<td>Present</td>
<td>2</td>
<td>Absent</td>
<td>Absent</td>
<td>Present</td>
<td>Absent</td>
<td>Absent</td>
<td>Spindle</td>
<td>Negative</td>
<td>Alive</td>
<td>112</td>
<td>KIT et al (1924A&gt;G, K642E)</td>
</tr>
<tr>
<td>9</td>
<td>69</td>
<td>M</td>
<td>Incisional biopsy</td>
<td>9.0</td>
<td>Absent</td>
<td>9</td>
<td>Absent</td>
<td>Present</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
<td>Spindle</td>
<td>N/A</td>
<td>Dead</td>
<td>52</td>
<td>None</td>
</tr>
<tr>
<td>10</td>
<td>52</td>
<td>M</td>
<td>Polypectomy</td>
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<td>Absent</td>
<td>0</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
<td>Lymphoma-like</td>
<td>N/A</td>
<td>Dead</td>
<td>21</td>
<td>None</td>
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<tr>
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<td>68</td>
<td>F</td>
<td>Local excision</td>
<td>1.1</td>
<td>Present</td>
<td>8</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
<td>Epithelioid &amp; lymphoma-like</td>
<td>Positive</td>
<td>Dead</td>
<td>87</td>
<td>None</td>
</tr>
<tr>
<td>12</td>
<td>46</td>
<td>M</td>
<td>Local excision</td>
<td>14.0</td>
<td>Present</td>
<td>6</td>
<td>Present</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
<td>Epithelioid &amp; spindle</td>
<td>Positive</td>
<td>Dead</td>
<td>21</td>
<td>None</td>
</tr>
<tr>
<td>13</td>
<td>65</td>
<td>M</td>
<td>Abdominoperineal resection</td>
<td>12.0</td>
<td>Absent</td>
<td>6</td>
<td>Absent</td>
<td>Present</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
<td>Epithelioid &amp; lymphoma-like</td>
<td>Negative</td>
<td>Dead</td>
<td>7</td>
<td>None</td>
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<tr>
<td>14</td>
<td>30</td>
<td>M</td>
<td>Abdominoperineal resection</td>
<td>11.0</td>
<td>Present</td>
<td>10</td>
<td>Absent</td>
<td>Present</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
<td>Spindle &amp; lymphoma-like</td>
<td>Positive</td>
<td>Dead</td>
<td>11</td>
<td>None</td>
</tr>
<tr>
<td>15</td>
<td>65</td>
<td>F</td>
<td>Local excision</td>
<td>4.5</td>
<td>Present</td>
<td>2</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
<td>Epithelioid &amp; spindle</td>
<td>N/A</td>
<td>Dead</td>
<td>6</td>
<td>None</td>
</tr>
</tbody>
</table>

N/A: Not applicable.
that consist entirely of tumor, and also due to ulceration and fragmentation in excisional biopsies. In our study, a third of the tumors were amelanotic and a third had an in situ component.

Microscopically, epithelioid, spindled, pleomorphic, and lymphoma-like tumor cells may co-exist, with epithelioid being the most frequent with combination of the others (32,40), similar to the present study. Therefore, AMs can mimic a large spectrum of malignancies, making the use of immunohistochemistry crucial in differential diagnosis. Additionally, lack of in situ component and/or lack of pigmentation, also complicate the diagnostic puzzle. At this point, an immunohistochemical panel of commonly used melanocytic markers, S-100 protein/ SOX10, Melan-A, HMB-45, can be helpful. Moreover, additional markers may be required to rule out other entities including primary or metastatic carcinomas, neuroendocrine neoplasms, sarcomas, lymphomas, and gastrointestinal stromal tumors. Among those, the use of CD117 requires careful interpretation due to its frequent positivity in AMs (up to 75% in the literature), which can lead to a misdiagnosis of rectal gastrointestinal stromal tumor, if not performed along with other melanocytic markers (12,33). Additionally, CD117 immunohistochemistry is known not to correlate with KIT status and therefore should not be used with mutation screening purposes (44).

In terms of pathological staging and prognosis, specific guidelines for reporting AMs do not exist. They are usually reported according to the American Joint Commission on Cancer (AJCC) guidelines for cutaneous melanoma (50), which depends mostly on tumor thickness, causing several issues in the daily practice. In the vast majority of cases on reported series (10,40,45,51-53) including ours (10.4 mm), the average tumor thickness was much thicker than the 4 mm threshold used for staging T4 cutaneous melanomas. This threshold inevitably categorizes the bulk of cases as T4, thus diminishing the prognostic stratification of the T classification. Several attempts have been made in order to sharpen the prognostic accuracy, including the implementation of different thickness cut-offs (51), subclassification depending on the localization (52) and metastatic status (40). Among other histopathologic prognostic factors, presence of metastasis, lymphovascular and perineural invasion, invasion of muscularis propria/anal sphincter were also reported (10,40,51). Mitotic rate is a very strong prognostic factor in cutaneous melanomas (54). Although high mitotic rates are frequently encountered similar to our study, their correlation with the clinical outcome is not well established in AMs (12,33). Nevertheless, studies on larger cohorts are needed in order to define the relationship between the distinct histopathologic parameters and prognosis.

In terms of treatment, optimal algorithms are lacking and satisfactory results are yet to be achieved (55). The primary choice of treatment is complete surgical removal of the tumor (8). Advantages of local approaches (mucosal resection or local excision) over extensive surgery (abdominoperineal resection) have long been discussed; however, literature data lack proof to recommend one modality over the other (56-58). Moreover, adjuvant or neoadjuvant therapies do not seem to make significant difference on the clinical outcome (55). The results of recently implemented immunotherapy is yet to be proven (59). Since our data involved limited information on adjuvant treatment, we were unable to draw any conclusions on this subject.

In conclusion, AMs are uncommon tumors with aggressive behavior and poor survival. They usually occur in the elderly and present in various gross and microscopic appearances, thus involving a wide spectrum of differential diagnoses. For accurate diagnosis, the melanocytic lineage should be demonstrated with immunohistochemistry, especially in the absence of conventional morphological clues such as pigmentation and/or in situ component. In terms of molecular profile, BRAF and KIT mutations rarely occur. Profiling of larger cohorts is required to elucidate the pathogenesis and to identify potential molecular indicators that may contribute in the development of individualized targeted therapies.

Conflict of Interest and Funding Statement

Authors have no conflicts of interest to declare. All authors have read and contributed to the final manuscript and confirm that this is an original work that has not been previously published, nor has it been submitted to another journal for simultaneous review. This study is supported by the Scientific Research Project Fund of Istanbul University (Project number: 51524). This study was partially presented in 28th Congress of the European Society of Pathology, 25-29 September 2016, Cologne, Germany.

Authorship Contributions

Concept: SOS, OT, Design: SOS, OT, Data collection or processing: OS, OT, IY, OH, Analysis or Interpretation: SOS, OT, IY, OH, Literature search: OT, OH, Writing: SOS, OT, OH, Approval: NB, MG.

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