Participation of hMLH1, p63, and MDM2 proteins in the pathogenesis of syndromic and nonsyndromic keratocystic odontogenic tumors

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Objectives. To evaluate the expression of hMLH1, p63, and MDM2 in Gorlin syndrome–associated keratocystic odontogenic tumors (SKOTs) and nonsyndromic keratocystic odontogenic tumors (NSKOTs).

Study Design. Seventeen primary NSKOTs, 17 SKOTs, and 8 recurrent NSKOTs were analyzed by using immunohistochemistry.

Results. No significant differences in the hMLH1, p63, or MDM2 labeling indices were observed between groups (P = .398; P = .232; P = .426, respectively). Higher hMLH1 immunoexpression was found in the basal layer of primary NSKOTs. Most KOTs exhibited p63 immunoexpression in the upper layers of the epithelium. MDM2 immunoexpression was observed in the upper epithelial layers of SKOTs and recurrent NSKOTs.

Conclusion. It was not possible to correlate the immunoexpression of hMLH1, p63, and MDM2 in SKOTs and primary and recurrent NSKOTs, suggesting that these proteins exert independent effects on the development of these groups of tumors.

Keratocystic odontogenic tumors (KOTs) are characterized by high rates of recurrence, infiltrative growth, and an occasional association with nevoid basal cell carcinoma syndrome (NBCCS). There are uncertainties as to whether differences exist in the biologic behavior of Gorlin syndrome–associated KOTs (SKOTs) and nonsyndromic KOTs (NSKOTs). In this respect, studies evaluating the proteins involved in cell cycle and apoptosis, as well as extracellular matrix components and proteases, support the existence of a different biologic behavior in SKOTs and NSKOTs.

Protein p63 plays an important role in epithelial development and in the proliferation of craniofacial structures. In the human oral mucosa, p63 is restricted mainly to the basal and parabasal layers of normal epithelium. Since cystic odontogenic lesions are of epithelial origin, p63 may play an important role in the growth and progression of these lesions.

The mismatch repair (MMR) system plays an important role in the maintenance of genetic stability. This system is involved in the removal of errors in DNA bases that arise during replication or as a result of DNA damage. Mutations in four genes of the MMR system (MSH2, hMLH1, MSH6, and PMS2) can influence the expression of p63 and MDM-2 and predispose to a variety of tumors.

According to Castrili et al., hMSH2 and hMLH1 are expressed in human cells that are undergoing rapid renewal; their reduced expression has been reported in several tumors. MDM2 protein, together with p53, participates in a complex regulatory network in response to genotoxic stress. Thus, defects in this process are related to the development of several types of cancer. Other genes in the TP53 gene family, such as TP63 and TP73, exert similar functions in tumor suppression, apoptosis induction, and cell cycle control.

Several studies have investigated the role of growth factors, tumor suppressor genes, and oncogenes in the onset and progression of KOT using immunohistochemistry. However, studies focusing on DNA repair proteins are sparse, and there are no studies explaining the participation of hMLH1 and its association with cell cycle

Statement of Clinical Relevance

This study contributes to the understanding of the biologic behavior of keratocystic odontogenic tumor (KOT). The association of hMLH1, MDM2, and p63 has been identified in colon cancer and head and neck carcinoma, and may provide additional insight into understanding the mechanisms related to the development and differences in behavior of KOT.
proteins in KOT. Based on the fact that microsatellite instability (MSI) is mainly caused by methylation-induced silencing of the *hMLH1* gene, we conducted the present study to evaluate whether differences in the behavior of KOTs are associated with changes in the expression of the *hMLH1* protein, in addition to p63 and MDM2, which can be influenced by defects in the production of this protein.

**MATERIALS AND METHODS**

The study was approved by the Ethics Committee of the Federal University of Rio Grande do Norte (UFRN), Natal, Brazil (No. 246.800). Seventeen cases of primary NSKOTs, 17 cases of SKOTs, and 8 cases of recurrent NSKOTs were retrieved from the archives of the Pathological Anatomy Services of the Departments of Oral Pathology of UFRN and University of Fortaleza (UNIFOR) for the period between January 2005 and December 2012. The number of available institutional archival cases defined the size of the sample. The histologic diagnosis was based on the third WHO classification in all cases.23 All patients with Gorlin syndrome had been diagnosed according to the criteria proposed by Evans et al.4 and had multiple KOTs. The patients with sporadic KOTs had single lesions and had been submitted to clinical and radiographic assessment to exclude the presence of other manifestations of Gorlin syndrome. All recurrent lesions were treated with surgical enucleation and did not correspond to recurrences from any of the primary NSKOTs in this study. Serial 3-μm-thick sections were cut from the tissue blocks and processed for immunohistochemical analysis.

**Immunohistochemistry**

The tissue sections were deparaffinized and immersed in 3% hydrogen peroxide to block endogenous peroxidase activity. The sections were then washed in phosphate-buffered saline (PBS). Antigen retrieval, antibody dilution, and clone type for p63, MDM2, and hMLH1 are shown in Table I. After treatment with normal serum, the sections were incubated with the primary antibodies in a moist chamber. Next, the sections were washed twice in PBS and treated with a polymer-based complex (Envision + Dual Link System — HRP; Dako, Carpinteria, CA) at room temperature to bind the primary antibodies. Peroxidase activity was visualized by immersing the tissue sections in diaminobenzidine (Liquid DAB+; Dako, Carpinteria, CA), which resulted in a brown reaction product. Finally, the sections were counterstained with Mayer’s hematoxylin and coverslipped. Sections of colon carcinoma served as positive controls for all antibodies. Samples treated as described above were used as negative controls; however, the primary antibody was replaced with a solution of bovine serum albumin in PBS.

**Analysis of immunostaining**

The tissue sections were examined in a blind fashion under an Olympus CX31 light microscope (Olympus Japan Co., Tokyo, Japan). The immunostaining for p63, MDM2, and hMLH1 was evaluated only in the epithelial lining of KOTs by establishing the labeling index. Briefly, tissue sections were examined at ×100 magnification to identify the area of the epithelial lining containing the largest number of immunostained cells. Next, 1000 epithelial cells were counted at ×400 magnification in consecutive fields throughout all epithelial layers. The p63, MDM2, and hMLH1 labeling indices are expressed as the percentage of immunostained nuclei in relation to the total number of nuclei counted. The intensity of the immunopositivity was not considered in the samples of the present study.

**Statistical analysis**

The results were analyzed using the Statistical Package for the Social Sciences (version 17.0; SPSS, Inc., Chicago, IL). The nonparametric Kruskal-Wallis test was applied to compare the percentage of p63-, MDM2-, and hMLH1-immunopositive cells in the epithelial lining among SKOTs, primary NSKOTs, and recurrent NSKOTs. The Spearman correlation test was used to determine possible correlations among the percentages of p63-, MDM2-, and hMLH1-immunopositive cells. A level of significance of 5% (∏ < .05) was adopted for all tests.

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**Table I. Properties of the primary antibodies**

<table>
<thead>
<tr>
<th>Clone</th>
<th>Specificity</th>
<th>Company</th>
<th>Dilution</th>
<th>Antigen retrieval</th>
<th>Incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 JUL</td>
<td>P63</td>
<td>Santa Cruz Biotechnology, Santa Cruz, CA</td>
<td>1:100</td>
<td>Citrate, pH 6.0, Pascal, 25° at room temperature</td>
<td>60 minutes</td>
</tr>
<tr>
<td>1 B10</td>
<td>MDM2</td>
<td>Novocastra Laboratories, Benton Lane, Newcastle upon Tyne, UK</td>
<td>1:50</td>
<td>Citrate, pH 6.0, Pascal, 15° in microwave</td>
<td>60 minutes</td>
</tr>
<tr>
<td>G168-15</td>
<td>MLH1</td>
<td>Dako, Carpinteria, CA</td>
<td>1:50</td>
<td>Tris-EDTA, pH 9.0, Pascal, and 25° at room temperature</td>
<td>18 hours</td>
</tr>
</tbody>
</table>
**RESULTS**

Immunoeexpression of hMLH1 in the epithelial component of KOT was positive in all cases analyzed. In primary NSKOT, immunostaining for hMLH1 was observed mainly in the basal epithelial layer (58.5%; n = 10). In SKOT and recurrent NSKOT, immunostaining was found in all layers of the epithelium, with no predominance in a specific layer. The median hMLH1 labeling index in the lining epithelium was 52.2 (range: 12.1-75.5) in the group of primary NSKOT, 56.9 (range: 23.3-69.7) in SKOT, and 41.2 (range: 10.8-69.0) in recurrent NSKOT. There was no significant difference between groups (P = .398, Kruskal-Wallis test) (Table II).

Immunoeexpression of p63 in the epithelial component of KOT was positive in all cases analyzed. Immunostaining predominated in the upper layers of the epithelium in 88.2% (n = 15) of primary NSKOT (see Figure 1B). In SKOT, immunoeexpression was detected in the basal and upper layers. Immunostaining predominated in the upper epithelial layers in 75% of recurrent NSKOT. The median p63 labeling index was 57.0 (range: 29.1-74.3) in the group of primary NSKOT, 61.7 (range: 23.6-76.0) in SKOT, and 50.2 (range: 37.8-79.5) in recurrent NSKOT. No significant difference was observed between groups (P = .232, Kruskal-Wallis test) (see Table II).

Immunoeexpression of MDM2 in the epithelial component of KOT was positive in all cases analyzed. In primary NSKOT, 76% (n = 13) exhibited immunostaining in the basal and upper layers of the epithelium. A predominance of immunostaining in the upper epithelial layers was observed in SKOT and recurrent NSKOT (see Figures 1G, 1H, and 1I). The median MDM2 labeling index in the epithelial lining was 61.9 (range: 31.7-91.5) for primary NSKOT, 57.0 (range: 25.9-78.6) for SKOT, and 54.35 (range: 13.8-75.9) for recurrent NSKOT. There was no significant difference between groups (P = .426, Kruskal-Wallis test) (see Table II).

Spearman’s correlation test showed no significant correlations among the p63, MDM2, and hMLH1 labeling indices in the epithelial component of KOT (P > .05).

**DISCUSSION**

The MMR repair system is important for maintaining genomic stability, reducing DNA errors, and preventing these errors from becoming fixed mutations during cell proliferation. MMR deficiencies are associated with a higher risk of development of different types of cancer. Immunohistochemical analysis of the expression of the *hMSH2* and *hMLH1* genes in tumors indicates that the expression of these genes is typically lost in patients with hereditary nonpolyposis colorectal cancer. Although the identification of mutations in the *PTCH* gene, among other factors, may support the classification of KOT as a neoplasm, there are no studies in the literature that define the role of the MMR system in these lesions. In the present study, no significant difference in the hMLH1 labeling indices was observed among primary NSKOTs, recurrent NSKOTs, and SKOTs (P > .05). Therefore, possible differences in the biologic behaviors of these lesions do not occur because of alterations in hMLH1 expression.

Castrili et al. showed that in ameloblastomas, hMSH2 and hMLH1 proteins are exclusively located in the nuclear compartment of the outer layer of the tumor nests. The restricted expression of hMSH2 and hMLH1 agrees with the probable stem-cell nature of the outer cell compartment from which inner stellate reticulum-like and squamous cells originate. The authors suggested that the development and progression of these tumors do not depend on defects in the human MMR system.
In primary NSKOT, immunopositive cells predominated in the basal epithelial layer, whereas immunoexpression was seen in all of the epithelial layers of SKOT and recurrent NSKOT. The present findings regarding the location of hMLH1 immunoexpression may indicate a more effective and consistent participation of the MMR system in primary KOT, preventing the perpetuation of DNA errors to the upper layers of the epithelium. On the other hand, the immunoexpression of hMLH1 in all of the epithelial layers of SKOT and recurrent NSKOT suggests greater genomic instability in these tumors compared with primary NSKOT. Moreover, the slight reduction in the hMLH1 labeling index observed in cases of recurrent NSKOT might be related to the loss of control of cell proliferation mechanisms, which explains the recurrence of these lesions.

The p63 gene expresses multiple isoforms. The most common isoform is p63α, which encodes the p63 protein, particularly ΔNp63α. This protein is abundant in the highly proliferative basal cells of adult epithelial tissues, including the oral epithelium. The p63 protein is also highly expressed the embryonic ectoderm, being essential in the differentiation of keratinocytes during embryonic development. Moreover, p63 participation in the proliferation and differentiation of epithelial cells has been suggested, since it is a homolog of the tumor suppressor p53.
With respect to the presence of p63 in KOT, this protein is thought to contribute to the biologic profile of these tumors, playing a role in the regulation of epithelial differentiation and favoring tumorigenesis.\textsuperscript{17}

Overexpression of p63 has been observed in the epithelial lining of KOT.\textsuperscript{3,17,18} In the present study, immunoexpression of p63 was observed in all cases studied, but there was no significant difference in the p63 labeling indices among primary NSKOT, recurrent NSKOT, and SKOT ($P > .05$). Similarly, Atarbashi-Moghadam et al.\textsuperscript{18} found no association between the expression of this protein in KOT and clinical features, such as recurrence and NBCCS.

With respect to the distribution of p63 immunoexpression in primary and recurrent NSKOT, higher expression was observed in the upper layers of the epithelium. The same has been reported in the literature for KOT, regardless of the type of tumor (isolated, recurrent, or syndromic).\textsuperscript{16-20} The immunoexpression of this protein in more superficial epithelial layers may be related to disturbances in cell cycle control, increasing the intrinsic growth potential. This fact would explain the infiltrative and aggressive growth of KOTs, as well as their higher recurrence rates, compared with other odontogenic lesions.\textsuperscript{3} In general, high p63 immunoexpression in KOT may reflect the immaturity of epithelial cells, favoring tumorigenesis, and therefore supports the hypothesis of a neoplastic origin of these tumors as well as the existence of a suprabasal proliferative compartment.\textsuperscript{17}

In most human cancers, the tumor suppressor function of p53 is compromised by mutations in the \textit{TP53} gene or by partial inhibition of the regulation of this protein. Under normal conditions, p53 levels are precisely regulated by MDM2.\textsuperscript{21} In the present study, MDM2 immunoexpression was observed in all KOT cases, in contrast to the literature, which reports immunoexpression in only 80\% of the cases studied,\textsuperscript{21} or even the complete absence of this marker in KOT.\textsuperscript{24} However, high MDM2 immunoreactivity in KOT cases compared with other odontogenic tumors has also been reported.\textsuperscript{22} Furthermore, in the present study, MDM2 immunoexpression predominated in the middle and upper compartments. This finding agrees with the observations of Sharifi-Sistani et al.,\textsuperscript{21} who compared the immunoexpression of MDM2 and p53 between KOT and ameloblastoma and found a correlation between these proteins, that is, an increase in one protein would lead to an increase in the other. The authors suggested that the increased immunoexpression of MDM2 in some tumors would not be able to reduce the amount of mutated p53.\textsuperscript{21}
The tumor suppressor gene functions of p53 have been shown to be mediated by the interaction with MMR genes and proteins. 14,26 DNA mismatch repair genes also play a role in the development of sporadic human neoplasms. Advances in our understanding of mismatch repair deficiency have important applications. Furthermore, the main function of MDM2 is to negatively regulate p53 by binding directly to the latter. This binding results in the formation of the MDM2/p53 complex, which inhibits the transcription activity of p53 and induces its degradation.26 However, no correlation among the hMLH1, p63, and MDM2 labeling indices was observed in the present study, suggesting that these proteins exert independent effects on the development of these tumors; in Figure 2, we have presented the pathway involving hMLH1, p63, and MDM-2. Future studies that include different members of DNA mismatch repair genes (hMSH2, hMSH6, and hPMS2) should be performed to further elucidate their role in the pathogenesis of these lesions.

REFERENCES


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