Original

Wingless-type Protein-1 (Wnt-1) Expression in Primary Conventional and Unicystic Ameloblastomas and Their Recurrent Tumors

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Abstract: Wingless-type protein (Wnt) is a family of 19 secreted glycoproteins that function as signaling transducers for cell-cell interaction, cell growth and differentiation. Wnt-1, a highly transforming member, has been implicated in tumorigenesis. The aim of this study was to elucidate the role of this cell signaling molecule in the development and progression of primary ameloblastoma and their recurrent tumors. Wnt-1 expression patterns were examined immunohistochemically in 22 primary and 14 recurrent ameloblastomas. These collectively consisted of the following subtypes: conventional (CA) (n=22), desmoplastic (DA) (n=2) and unicystic (UA) (n=12) ameloblastoma. Results demonstrated that CA (n=20/22; 90.9%), DA (n=2/2; 100%) and UA (11/12; 91.7%) showed high Wnt-1 expression percentages. Strong staining intensity for Wnt-1 was observed more frequently in primary CA (n=10/13; 76.9%) than in their recurrent counterparts (n=2/9; 22.2%) (p < 0.05). Conversely, in UA, recurrent tumors (n=3/5; 60.0%) tend to stain strongly for Wnt-1 more frequently than their primary lesions (n=3/7; 42.9%) (p > 0.05). Keratinizing cells in areas of squamous metaplasia also expressed Wnt-1 more intensely compared to their surrounding polyhedral stellate reticulum-like cells. Tumor islands containing granular cells were also Wnt-1 positive. Present findings confirmed that the Wnt signaling pathway is activated in ameloblastoma. Strong Wnt-1 expression in primary conventional and recurrent unicystic ameloblastoma suggests that Wnt-1 plays a more critical role in these subtypes. Positive expression of Wnt-1 in keratinizing ameloblastomatous tumor epithelium and granular cells complies with the anti-apoptotic properties of Wnt-1. Negative reactivity for Wnt-1 in 3 cases of ameloblastoma suggests that the development and progression of ameloblastoma may occur independent of this cell signaling molecule.

Key words: Ameloblastoma, Immunohistochemistry, Wnt-1

Introduction

Ameloblastoma is a histological benign, but locally aggressive tumor arising from the odontogenic ectoderm and its derivatives. According to the World Health Organization, ameloblastoma is classified into four clinicopathological subtypes: unicystic, conventional (solid/multicystic), peripheral and malignant1). Histologically, there are two basic growth patterns, follicular and plexiform.

Wingless-type protein-1 (Wnt-1), belongs to a large family of 19 secreted glycoproteins that act as signaling transducers involved in cell proliferation and development2). Wnt-1 proteins signal via the Wnt/canonical pathway. There is evidence that this signaling pathway plays a critical role in tumorigenesis2-3). Previous studies have shown Wnt-1 overexpression in human cancers including basal cell carcinoma4), oral squamous cell carcinoma5), non-small cell lung cancer6) and mammary cancer7). The development and progression of ameloblastoma is a complex and ill-understand process. Many genes involved in tooth development too play critical roles in the tumorigenesis of ameloblastoma8-14). The canonical Wnt signaling molecules play an essential role during tooth development15-16). Thus far Wnts-
-3, -4, -5a, -6, -7b and 10b expressions during tooth development from the earliest formation of epithelial thickenings to early bell stage have been clarified. Some of these cell signaling molecules namely Wnt-1, Wnt-2, Wnt-5a, beta-catenin, APC, AXIN1 and AXIN2 have been examined in ameloblastoma. The aim of this study was to determine the immunolocalization of Wnt-1 in ameloblastomas in terms of its distribution and staining intensity in primary conventional and unicystic ameloblastomas and their recurrent tumors, and to speculate on the relevance of these findings in relation to the development and progression of this neoplasm.

**Materials and methods**

**Tissue samples**

The tissue samples in this study were from the surgical pathology files of the Department of Oral Pathology, Oral Medicine & Periodontology, Faculty of Dentistry, University of Malaya, Kuala Lumpur, Malaysia. Thirty-six cases comprising 22 conventional, 2 desmoplastic and 12 unicystic ameloblastomas (totaling 22 primary and 14 recurrent cases) were retrieved. These cases were reviewed and selected according to the World Health Organization Classification of Tumors (Barnes et al.).

The clinical characteristics namely age, gender and tumor location of these cases were recorded.

From the archival formalin-fixed, paraffin-embedded tissue blocks of these cases new 5μm thick sections were prepared for staining with hematoxylin-eosin, and for immunohistochemistry with rabbit anti-Wnt-1 polyclonal antibody (Genetex Inc., USA).

**Immunohistochemistry**

The Envision technique was used for the immunohistochemical detection of Wnt-1. Briefly, deparaffinized sections of 5μm thickness were pretreated for antigen retrieval by microwaving (99°C) in 10 nM of citrate buffer (pH 6, 20 min). These sections were then immersed in 0.3% methanol containing 3% hydrogen peroxide for 20 min, to block endogenous peroxidase, and rinsed in 0.05 M Tris-buffered saline (TBS) (5 min, two times) before immersing in blocking solution (Dako, CA, USA) for 20 min at room temperature. Then the sections were covered with primary antibody (rabbit anti-human Wnt-1 at 1:150 dilution; Genetex Inc., USA) and incubated for 1 h at room temperature. Immunoreactions were performed using Envision Kit (Dako, CA, USA). The antigenic sites were visualized using diaminobenzidine (DAB) substrate chromogen (Dako, CA, USA) and counterstained with Mayer’s hematoxylin. For negative control, sections were treated as above but without the primary antibody. All the control sections were negative. Positive staining control was also included.

**Staining intensity**

The tumors were analyzed subjectively according to the proportion of immunoreactive tumor cells (pre-ameloblast and stellate reticulum-like cells) and intensity of Wnt-1 staining, and were categorized as follows: negative, no staining of tumor; weakly positive, staining is faintly present in focal areas (<25%); moderately positive, staining is evident in large parts of tumor (25-50%); and strongly positive, pronounced staining is present in large parts of the tumor (>50%).

**Statistical analysis**

Chi square test was performed to compare the expression level...
of Wnt-1 with the clinicopathological subtypes of ameloblastoma. Level of significance was set at $p<0.05$.

Results

Clinical findings

The clinical characteristics of the cases in the study sample are detailed in Table 1. There were 23 (63.9%) male and 13 (36.1%) female patients with an overall mean age of 27.4 years (age range: 11-57 years). Their racial composition was 50.0% Chinese, 33.3% Malays, 11.1% Indians and 5.6% other races. Thirty-three cases (91.7%) were from the mandible and the remaining 3 (8.3%) cases were from the maxilla.

Immunohistochemical findings

The immunohistochemical results and grades of staining intensity are summarized in Table 2 and illustrated in Figs. 1-3. Wnt-1 expression was detected in 33 (91.7%) out of 36 cases of ameloblastoma. Their localization patterns according to the subtypes are detailed below.

Conventional ameloblastoma ($n=22$)

Ten cases of plexiform, 8 follicular and 4 mixed plexiform-follicular ameloblastomas were examined (Table 2 and Fig. 2, A-D). Wnt-1 of variable intensity was detected in the cell membrane and cytoplasm of pre-ameloblast and stellate reticulum-like cells in those cases expressing Wnt-1 positivity. Keratinization in areas of squamous metaplasia and granular cells stained for Wnt-1 (Fig. 3, A-C). Strong Wnt-1 immunoreactivity was scored more frequently in primary conventional ameloblastoma ($n=10/13$, 76.9%) than in their recurrent tumors ($n=2/9$, 22.2%). The difference was statistically significant ($p<0.05$). Both cases of Wnt-1-negative conventional ameloblastoma were of follicular pattern ($n=2/8$, 25.0%) (Fig. 3, E-F).

Desmoplastic ameloblastoma ($n=2$)

Both cases stained positive for Wnt-1 (Table 2 and Fig. 3, D). Wnt-1 activity was variably localized in the cell membrane and cytoplasm of both peripheral and central tumor epithelial cells.

Unicystic ameloblastoma ($n=12$)

Five cases each of simple and mural unicystic ameloblastoma, and 2 cases of plexiform unicystic ameloblastoma were examined (Table 2 and Fig. 2, E-F). Strong Wnt-1 expression was observed more frequently in recurrent lesions ($n=3/5$; 60.0%) than in the primary tumors ($n=3/7$; 42.9%). The difference was statistically not significant ($p>0.05$). Wnt-1 expression ranged from negative to moderately–intense for stromal cells and extracellular matrix in all cases of ameloblastoma examined.

Discussion

The Wnt gene family encodes 19 cysteine-rich signaling molecules and many of these are involved in diverse cellular processes such as cell growth, cell fate and motility. The first suggestion of Wnt signaling pathway in cancer development was made more than 27 years ago following the discovery of Wnt-1, the first member of the Wnt family, as an integration site for mouse
mammary tumor virus in mouse mammary carcinoma\textsuperscript{21}. It is classified as a highly transforming Wnt by virtue of its high transforming ability to promote tumor progression. Mutations of Wnt genes may cause inappropriate activation of the Wnt signaling pathways leading to tumor development and progression\textsuperscript{22-23}. Strong genetic evidence from studies in mice, \textit{Drosophila} and \textit{Xenopus} suggests a critical role of Wnt-1 in induced tumorigenesis. In particular, Wnt-1 has been implicated in the abnormal proliferation of human tissues. Aberrant levels of Wnt-1 have been detected in a variety of human tumors and cancers including oral squamous cell carcinoma\textsuperscript{5}, basal cell carcinoma\textsuperscript{4}, non small cell lung cancer\textsuperscript{6} and mammary cancer\textsuperscript{7}.

Cell signaling molecules have also been examined in ameloblastoma. Wnt-1 protein has been detected in the epithelial tumor cells of intraosseous ameloblastoma of follicular, acanthomatous, desmoplastic and plexiform subtypes\textsuperscript{17}. Strong expression of beta-catenin and Wnt-5a and lack of reactivity for Wnt-2 was demonstrated in granular cell ameloblastoma\textsuperscript{18}. Activation of Wnt signaling pathway through demonstration of nuclear beta-catenin accumulations and mutations of AXIN1 and AXIN2 have also been documented\textsuperscript{20}.

The purpose of this study was to characterize primary and recurrent ameloblastoma based on variations in protein expression patterns derived from immunohistochemical analysis of Wnt-1. To fulfill this purpose, a total of 22 primary and 14 recurrent ameloblastomas were examined with regards to the immunolocalization and intensity of reactivity of Wnt-1 in the various neoplastic cellular components of ameloblastoma. Our results showed that Wnt-1 expression was upregulated in 33 (91.7%) out of 36 cases of ameloblastoma. This score is considerably higher than a previous study\textsuperscript{17} where 19 (65.5%) out of 29 ameloblastoma tumor samples reportedly expressed Wnt-1. The disparity in findings\textsuperscript{17} may be due to two main reasons. Firstly, differences in the spectrum of ameloblastoma subtypes analyzed between the earlier study\textsuperscript{17} and the current series may account for the difference in the percentages of Wnt-1 positive tumors observed (variation in Wnt-1 expression profile and ameloblastoma subtypes will be discussed later). In the earlier study\textsuperscript{17} there were 13 follicular, 9 plexiform, 3 acanthomatous and 4 desmoplastic ameloblastoma whereas the present series comprised 8 follicular, 10 plexiform, 4 mixed plexiform-follicular, 2 desmoplastic and 12 unicystic (5 simple, 5 mural and 2

Figure 2 Photomicrographs showing H&E staining and immunolocalization of Wnt-1 in plexiform (A, B), follicular (C, D) and unicystic (E, F) ameloblastomas. (A, C, E: H&E; x100; B, D, F: anti-Wnt-1; x100).
plexiform) ameloblastomas. Secondly, it was not known whether the ameloblastoma tumor sample evaluated represented primary or recurrent tumors. This factor may influence to a certain extent the expression profile for Wnt-1 (to be discussed later). The Wnt signaling mechanism can be activated through 3 distinct pathways namely the canonical/β-catenin, planar polarity and the Wnt/ Ca²⁺ pathway. As Wnt-1 is an inducer of the canonical/β-catenin pathway, a high expression for Wnt-1 in ameloblastoma implies that the Wnt signaling pathway is activated in this neoplasm. It also confirms the findings of an earlier study that demonstrated activation of Wnt signaling pathway through the detection of â-catenin and APC gene protein in ameloblastoma. This is because Wnt interaction with its Freezle receptor activates this mechanism via the canonical/β-catenin pathway leading to the accumulation of â-catenin initially in the cytoplasm and subsequently translocated to the nucleus.

In the present series, the loss of expression for Wnt-1 in the remaining 3 cases (2 follicular and 1 simple unicystic ameloblastomas) suggests that tumorigenesis in the ameloblastoma may occur independent of Wnt-1 activity. Wnt-1-independent tumorigenesis has also been reported in some cases of colorectal cancers.

It is well-known that ameloblastoma is a benign but locally-invasive odontogenic epithelial neoplasm which has a tendency to recur even after many years of apparent cure. It is also known that the conventional ameloblastoma is a biologically more aggressive tumor in terms of its local behavior and potential for recurrence than the unicystic variant. Therefore it seems prudent to determine how useful is the Wnt-1 protein molecule as a marker of tumor behavior by evaluating whether any differences exist in the expression pattern for Wnt-1 between conventional and unicystic ameloblastoma. In the present study, 10 (76.9%) cases of primary conventional ameloblastoma demonstrated strong Wnt-1 immunoreactivity, and this may denote that this ameloblastoma subtype often tends towards an increased cell proliferation. In contrast only 3 (42.9%) cases of primary unicystic ameloblastoma strongly expressed Wnt-1. Due to the smallness of the number of unicystic ameloblastoma evaluated, additional studies are needed to investigate this issue further.
A disparity in the intensity of Wnt-1 expression was also demonstrated between primary and recurrent tumors of both the conventional and unicystic ameloblastoma. Only 2 (22.2%) cases of recurrent conventional ameloblastoma compared to 3 (60.0%) cases of recurrent unicystic ameloblastoma strongly for Wnt-1. This heterogeneous pattern of Wnt-1 expression suggests that it is conceivable that different signaling proteins or members of the Wnt family (other than the Wnt-1 glycoprotein) may be variously involved in the development and progression of primary and recurrent lesions.

Wnt-1 express its oncogenic potential via an anti-apoptotic mechanism\(^{20}\). In Rat-1 cells, overexpression of Wnt-1 inhibits apoptosis by activating β-catenin/T-cell factor-mediated transcription\(^{21}\). In this study, a distinct observation was that pre-ameloblast-like cells tend to show stronger Wnt-1 expression compared to the stellate reticulum-like cells. Tumor islands of ameloblastoma purportedly have an anti-apoptotic proliferating site in the peripheral layer and a pro-apoptotic site in the central layer\(^{22}\). Similarly keratinizing and granular cells within the central layer were also viewed as pro-apoptotic sites\(^{23}\). In the current study, we observed that there was a tendency for granular cells within ameloblastoma tumor islands and keratinizing cells in areas of squamous metaplasia to show stronger Wnt-1 immunoreactivity compared to the surrounding polyhedral or stellate reticulum-like cells. This finding suggests that Wnt-1 anti-apoptotic mechanism probably plays a greater role in keratinizing and granular cells compared to the central stellate reticulum-like cells\(^{24}\). The two cases of desmoplastic ameloblastoma too demonstrated moderately intense staining for Wnt-1 in their tumor islands. This preferential Wnt-1 expression in certain ameloblastoma cell types suggests that Wnt-1 activity differs in the different cellular neoplastic components. As our series of ameloblastoma did not include cases showing basal or clear cell change, we were unable to confirm the role of Wnt-1 in the cellular characterization of these histological variants.

In summary, Wnt-1 protein expression was evaluated in 36 cases of ameloblastoma. Results showed a high percentage (91.89%) of ameloblastoma expressed Wnt-1 suggesting activation of the Wnt signaling mechanism via the classical/canonical pathway. Distinct differences were observed in the Wnt-1 expression profile between conventional and unicystic ameloblastoma. Similarly, differences in Wnt-1 expression intensity were found between primary and recurrent tumors within each subtype. Pre-ameloblast-like cells stained more strongly for Wnt-1 than the stellate reticulum-like cells suggesting that Wnt-1 plays a role in the cellular characterization of these cells.

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