Keratocystic Odontogenic Tumors: Predictive Factors of Recurrence by Ki-67 and AgNOR Labelling

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Abstract

Purpose: The purpose of the present study was to investigate the possible role of Ki-67 and argentophilic nucleolar organizing regions (AgNOR) between the recurrent and nonrecurrent keratocystic odontogenic tumors (KCOTs). Another aim was to compare the correlation between these two markers.

Materials and Methods: 22 KCOTs were evaluated retrospectively. The actual proliferative activity of the KCOT was measured by Ki-67 labelling index and argentophilic nucleolar organizing regions AgNOR count per nucleus. Results: Recurrence occurred in 3 patients (13.6%) during the follow-up period (mean follow-up, 37.8 months) The Ki-67 and AgNOR counts were significantly higher in the recurrent lesions comparing to the non-recurrent lesions. (p=0.045; p=0.049) The correlation between Ki-67 and AgNOR counts was found to be positive (r=0.853 p=0.0001).

Conclusion: Within the limit of the present study, it is thought that Ki-67 and AgNOR might be helpful as a prognostic marker for the recurrences of KCOTs. These markers reinforced the meaning of the new classification of the lesion as an odontogenic tumor. Enucleation with curettage or decompression following enucleation with curettage is a simple and appropriate surgical model for the treatment of KCOT despite the relative high recurrence rate. On the other hand, the conservative treatment can be chosen only if there is no coronoid invasion, no interruptive cortical lysis and no tissular invasion.

Key words: keratocystic odontogenic tumors, Ki-67, AgNOR

Introduction

Keratocystic odontogenic tumor (KCOT) defined by the World Health Organization (WHO), is a benign, intraosseous neoplasm of dental origin, with a characteristic lining of parakeratinized stratified squamous epithelium (1). Increased activity of the epithelium, has been confirmed by previous studies that have compared KCOTs with other odontogenic cysts may explain the high recurrence rates of KCOTs. Some are associated with nevoid basal cell carcinoma syndrome (2).

Immunohistochemical studies have examined KCOTs by using various markers of proliferation and of apoptosis. The proliferative activity of the epithelial lining of KCOTs has been the subject of various investigations by using different markers of proliferation as Ki-67 (2). Ki-67 is the prototypic cell cycle related nuclear protein, expressed by proliferating cells in all phases of the active cell cycle. It rapidly degrades after mitosis with a half-life of detectable antigen being an hour or less (3). Immunohistochemical
detection of Ki-67 has been used to evaluate the proliferative potential of healthy cells as well as of preneoplastic and neoplastic lesions (4).

Nucleolar organizer regions (NORs) are loops of DNA which transcribes to ribosomal RNA. The NOR-related protein becomes visible in nucleus by a silver-staining technique under a light microscope, and it has been named argyrophilic protein of NOR (Ag-NOR) (5). Several different methods have been proposed to determine the proliferative rate in tumors. Silver staining of AgNORs is considered to be the best and most cost-effective marker to assess the proliferative behaviour of a lesion. The rapidity of the cell turnover is evaluated by speed of the cell cycle, so as to assess the growth rate of the lesion, which is easily assessed by AgNOR count per nucleus. The amount of AgNOR represents a cell kinetics parameter and can be used for prognostic purposes. AgNOR counts have been demonstrated to offer a predictive index in various malignancies (6,7).

Pathologists’ interest in AgNOR proteins increased greatly around the end of 1980s following the observation that malignant cells frequently exhibit a greater AgNOR protein amount as compared with the corresponding benign or normal cells. AgNOR method has become widespread among pathologists, in various fields of tumour pathology (8).

The objective of the present study was to investigate clinical behavior of KCOTs regarding the recurrence, by evaluating Ki-67 and AgNOR staining. Another purpose was to investigate the correlation between these two markers and to demonstrate their possible prognostic role in these lesions.

Material and Methods

Tissue Samples

In the present study, KCOTs treated by two oral and maxillofacial surgeons from January 2004 to August 2010, were reviewed retrospectively. Clinical and histological information was recorded for each patient. The inclusion criteria of the study was the histopathological diagnosis of “KCOT”. The cases which were previously treated in another service were excluded. The cases followed-up shorter than 1 year and which were associated with nevoid basal cell carcinoma syndrome (NBCCS) were also excluded from the study. The histopathological diagnosis was based on the criteria showing parakeratinization of the lining epithelium as described by WHO guidelines (1).

According to these criteria, a total of 22 histopathologically diagnosed KCOTs were selected for the study. Routinely panoramic radiographs were used before the operation. CT scans were also used according to the clinical features (size, anatomical location) of the tumor. The data included; age at diagnosis, gender of the patients, location of the lesion, clinical manifestation (pain, swelling, infection) treatment modality and recurrence (Table 1).

All subjects were evaluated clinically and radiographically at regular times. Panoramic radiographs were taken at 6 and 12 in the first post-operative year followed by once every year. The average follow-up period was 37.8 months.

Immunohistochemical staining

For immunohistochemistry, the parafin blocks were cut serially into approximately 5 μm thick sections on charged slides. Firstly, the sections were penetrated and dried overnight in an autoclave (56°C). They were deparaffinized with xylene for 30 min, washed with 99% alcohol for 15 minutes, and then with 96% alcohol and distilled water. Histostain-Plus Bulk Kit (Zymed 2nd Generation, LAB-SA Detection System, 85-9043) was used in this study. For antigen retrieval, the sections were microwaved four times for 5 min in citrate buffer (Ph 6.0). Endogenous peroxidase activity was blocked by incubating the sections with 3% H2O2 and washed with distilled water and waited in PBS for 5 min. To prevent non-specific reactions, sections were incubated with block solution. Ki-67 with a dilution ratio of 1:50 (Zymed Laboratories, Mouse, Monoclonal, Clone 7B11) was used as the primary antibody. Slides were incubated with Ki-67 for 120 min. The secondary antibody was reacted for 25 min. AEC (Zymed Laboratories, 00-2007, Lot No:319293A) chromogen was used to visualize the reaction. Finally, the sections were counterstained with Mayer’s hematoxylin, coverslipped and evaluated by a light microscope.

AgNOR staining

For AgNOR staining, the paraffin blocks were cut into approximately 5 μm thick sections. The sections were deparaffinised with xylene for 30 minutes, and washed with 99% alcohol for 15 minutes, then 96% alcohol and distilled water. The slides were immersed in a citric acid solution/ethanol solution (1:3). The 50% silver nitrate solution was mixed in 2 g/dL gelatin solution dissolved in 1g/dL formic acid in a 1:2 proportion and the sections were incubated at a dark room for 30 min. After washing off the distilled water, the sections were dehydrated with ethanol, cleared with xylene, and coverslipped and evaluated by a light microscope.
Table 1: Patient demographics and characteristics of the lesions with Ki-67 and Ag NOR.

| Gender | Age | Location                        | Follow-up (months) | Treatment method                                      | Recurrence | Ki-67 | Ag-NOR |
|--------|-----|---------------------------------|--------------------|-------------------------------------------------------|------------|-------|--------|
| M      | 68  | Mandible anterior               | 34                 | decompression and enucleation with curettage           | -          | 4     | 3,60   |
| M      | 25  | Mandible posterior              | 17                 | enucleation with curettage                             | -          | 3,5   | 2,70   |
| M      | 55  | Mandible posterior              | 16                 | enucleation with curettage                             | -          | 3     | 3,30   |
| M      | 58  | Mandible premolar and anterior  | 46                 | enucleation with curettage                             | -          | 2,8   | 2,20   |
| M      | 65  | Mandible premolar and posterior | 65                 | enucleation with curettage                             | -          | 2,3   | 3,10   |
| F      | 51  | Maxilla premolar and anterior   | 41                 | decompression and enucleation with curettage           | -          | 4     | 3,51   |
| M      | 50  | Mandible posterior              | 38                 | enucleation with curettage                             | -          | 4     | 3,60   |
| M      | 50  | Mandible premolar and anterior  | 38                 | enucleation with curettage                             | -          | 5,5   | 4,60   |
| M      | 37  | Maxilla, posterior, premolar,   | 37                 | enucleation with curettage                             | +          | 4,5   | 4,00   |
| F      | 69  | Mandible premolar               | 41                 | enucleation with curettage                             | -          | 4     | 3,90   |
| M      | 24  | Maxilla premolar, posterior     | 64                 | enucleation with curettage                             | +          | 4     | 4,10   |
| M      | 50  | Mandible posterior              | 19                 | enucleation with curettage                             | -          | 4     | 3,80   |
| F      | 40  | Mandible posterior              | 20                 | enucleation with curettage                             | -          | 4,5   | 4,30   |
| M      | 62  | Mandible premolar               | 12                 | enucleation with curettage                             | -          | 4,5   | 4,40   |
| M      | 56  | Mandible premolar               | 72                 | enucleation with curettage                             | -          | 5     | 4,45   |
| M      | 53  | Maxilla, posterior, premolar,   | 53                 | enucleation with curettage                             | -          | 2,5   | 3,20   |
| M      | 39  | Mandible posterior, premolar,   | 38                 | enucleation with curettage                             | -          | 3     | 3,33   |
| F      | 49  | Mandible posterior              | 39                 | enucleation with curettage                             | -          | 3     | 4,50   |
| M      | 58  | Mandible posterior              | 38                 | enucleation with curettage                             | -          | 3     | 3,70   |
| F      | 21  | Maxilla anterior                | 34                 | enucleation with curettage                             | -          | 3,5   | 3,30   |

Evaluation Methods

Ki-67 immunostained slides were examined at 400x magnification in Olympus BX60 microscope. In epithelium, the positive and negative cells were counted in 5 contiguous and consecutive microscopic high-power fields. The number of positive cells was divided into the total number of cells counted in the whole layer. The result was multiplied by 100 to find the percentage of positive cells. AgNOR-stained slides were examined at 1000x magnification with immersion oil in Olympus BX60 microscope. The number of AgNORs in the nucleus was counted in 250 cells for each case. Black dots/aggregated clusters within cellular nucleoli were counted as one dot. The average number of AgNORs was divided into total number of cells.

Statistical analysis

Statistical analysis was aided by the NCSS (Number Cruncher Statistical System) 2007 Statistical Software (Utah, USA). Chi-square test and Fisher's exact test were used to assess qualitative parameters. Mann-Whitney-U test was used to evaluate descriptive statistics (median, interquartile range) (SD) and differences between groups. Probabilities of less than 0.05 were accepted as significant.

Results

The ages of recurrent group members were found to be statistically lower than the non recurrent group members (p=0.035). No statistically significant difference was observed between the recurrent and non recurrent groups regarding the follow-up. (p=0.472). Ki-67 values of recurrent group were found
to be statistically higher than the non-recurrent group. (p=0.045). AgNOR values of recurrent group were found to be statistically higher than the non-recurrent group (p=0.049) (Table 2).

**Table 2: Comparison of recurrent and non recurrent lesions.**

|                      | Non recurrent | Recurrent | MW  | p    |
|----------------------|--------------|-----------|-----|------|
| **Median (IQR)**     |              |           |     |      |
| Age                  | 51 (40-58)   | 32 (24-37)| 6.5 | 0.035|
| Follow-up            | 38 (34-50)   | 42 (37-64)| 21  | 0.472|
| KI-67                | 3.5 (3-4)    | 4.5 (4-6) | 8   | 0.045|
| AgNOR                | 3.6 (3.3-3.9)| 4.1 (4-5.5)| 8   | 0.049|

Most of the Ki-67 cells being detected in the suprabasal layers (Figure 1). The AgNOR dots were higher in the nucleus of subrabasal cells than basal cells in KCOT (Figure 2).

No statistically significant difference was observed between the recurrent and non recurrent groups regarding the gender (p=0.952).

No statistically significant difference was observed between KI-67 and AgNOR regarding the age range.

Statistically significant positive correlation was found between KI-67 and AgNOR values. (r=0.853 p=0.001). (Table 3).

**Figure 1:** Representative photomicrograph of keratocystic odontogenic tumor stained with Ki-67. Immunoreactivity was observed particularly in the suprabasal cell layers of lining epithelium. (x400)

**Figure 2:** Representative photomicrograph of argyrophilic nucleolar organizing regions (AgNOR) staining. The AgNOR dots were higher in the nucleus of subrabasal cells than basal cells in keratocystic odontogenic tumour. (x1000)

**Table 3: The correlation between AgNOR and KI-67.**

| AgNOR | r          | p         |
|-------|------------|-----------|
|       | 0.853      | 0.0001    |

**Discussion**

In our study, no statistically significant difference was observed between the recurrent and non recurrent groups regarding the gender; although some authors consider that gender could play an important role in the recurrence rate (9,10). Others consider that the gender is not a significant determinant of recurrence (11,12,13).

The ages of recurrent group members were found to be statistically lower than the non-recurrent group members (p=0.035). This situation was similar to that found by Forssell et al and Gonzalez-Alva et al. who reported a higher recurrence rate in young patients (14, 15). This situation may be associated that younger patients often receive more conservative approach such as preserving the associated teeth as presented in our study.

Conservative and aggressive approaches have been reported about the management of KCOT. The choice of the treatment must take into account the patient’s age, size of the lesion, previous recurrence history, soft tissue involvement and histological characteristics (16). In the conservative method, simple enucleation with or without curettage, marsupialization and decompression are suggested. Aggres-
sive methods include; peripheral ostectomy, chemical curettage with Carnoy’s solution, and periost, tissue or bone radical resection (17, 18). The treatment of KCOTs is still controversial. A recent Cochrane re-
view demonstrated that there is a need for well con-
ducted randomized controlled clinical trials about the
management of the KCOTs (19).

The recurrence rates of reported KCOTs tend to vary from 0% to 100% (2, 18, 20, 21). These marked discrepancies are thought to be related to the different lengths of postoperative follow-up periods, operative techniques employed or inclusion of cases with ne-
void basal cell carcinoma syndrome (NBCCS). Tu-
ors also vary in their aggressiveness, contributing to
variation in recurrence patterns (18). Recurrence rate is highest with the simple enucleation (without cu-
rettage) and range from 9% and 62.5% (22). Resection offers a high cure rate, but produces significant mor-
bidity such as the loss of the jaw continuity or facial disfigurement. It should therefore be reserved only
for aggressive or recurrent lesions, or for patients who
cannot be closely followed up after conservative

treatments (22). In our study, all the cases were
treated with enucleation with curettage as reported
Boffano et al (23). A surgical burr was used to remove
about 2 mm of cortical bone all around the remained
bone after enucleation. Only in 2 cases decompression
was performed before the enucleation with curettage.
The recurrence rate was 13.6%; as similar with the
reports which showed the enucleation and curettage
as a treatment of KCOT, as presented in our study (18,
23). On the other hand the recurrence rate in our
study was found to be as rather low compared with
previous reports which demonstrated the simple
enucleation method as a treatment of KCOT (24,25,26,
27).

The patients are still under periodic observation
in our institution. On the other it has been reported
that KCOT may recur even after 6 to 25 years after
enucleation (25). Therefore it is not possible to draw
any long-term conclusions on recurrence rates re-
garding the mean follow-up time (37.8 months) of the
present study.

It has been reported that KCOTs which were lo-
cated in the mandibular molar region had signifi-
cantly higher recurrence rates than those in other
sites. Difficulty in removing all the traces of the epi-
thelial lining is believed to be a major factor leading to
recurrence (17). In our study, only one of the recurrent
lesion was observed in the mandible (no 7 in table 1).
The lesion size was more than 5 cm and located in the
posterior part of the mandible, raising up to the con-
dyular process. After 8 months of decompression the
tumor was enucleated with curettage. 11 months after
enucleation, the lesion recurred and treated again
within the same conservative approach. The recur-
rence was associated with the difficult surgical access,
large size and also biological aggressive behaviour
of the lesion itself which was demonstrated with the
highest Ki67 label index and AgNORs counts. It was
not possible to remove the tumour in one piece. The
other two recurrent lesions were seen in the maxilla.
The lesions sizes were more than 4 cm and the lesions
were removed in one piece. The sizes of the
non-recurrent tumors vary from 1 to 5 cm. The recur-
rence may be explained with the conservative treat-
ment (root resection) of the associated teeth. Extrak-
tion of teeth has been kept to a minimum. The patients

denied multiple teeth extractions and accepted the
risk of recurrence. Therefore it is thought that some
tumor remnants which were associated with the teeth
may cause the recurrence as reported Pogrel (28).

KCOTs arise from odontogenic epithelial cells and
remnants of the dental lamina, and also from ex-
tensions of basal cells of the oral epithelium. Epithelial
cells in KCOTs seem to have a different proliferative
potential from those of other odontogenic lesions (4).

Ki-67 antibodies are useful in establishing the
cell growing fraction in neoplasms. Ki-67 expression
has been shown to be higher in the epithelium of
KCOTs when compared with developmental and in-
flammatory cysts, with most of the Ki-67 cells being
detected in the suprabasal layers, as reported in our
study (2, 3, 29).

Limited numbers of the studies were published
about the evaluation of AgNORs in odontogenic cysts
and tumors. Conflicting results have been observed in
these studies (6, 30,31,32,33,34). Coleman et al. in-
vested whether AgNORs may be of value in distin-
guishing various odontogenic cysts from the unicystic
ameloblastoma. They concluded that AgNOR counts
are not of diagnostic significance and can not be used
to distinguish the various odontogenic cysts from one
another nor from the unicystic ameloblastoma (30).
Allison and Spencer performed AgNOR counts on
apical periodontal cysts, dentigerous cysts, odonto-
genic keratocysts, ameloblastomas and basal cell car-
cinomas. They reported significant differences be-
tween KCOT and apical periodontal cyst. In that
study the mean AgNOR counts for all odontogenic
cysts ranged between 2.02 and 2.65, and for amelo-
blastomas were 2.24. According the results they con-
cluded that the method had neither a diagnostic nor a
prognostic value in these lesions (34). In our study, the
AgNOR dots were higher in the nucleus of
subrabasal cells than basal cells in KCOT. The mean
values of AgNORs were found higher regarding the
count range reported in the literature (6, 32, 34).
The differences in the literature may be associated with the differences in staining method and counting protocol for AgNOR and because of the difference in simple size. Therefore we are agree with Gadball et al and Eslami et al who recommended a standard protocol for AgNOR staining and counting protocol (6, 32).

There are several confounding factors for the analysis of relationship between recurrence of KCOT and clinicopathologic and immunohistochemical variables. There is no doubt that the surgical management is the most influential factor for the recurrences following any surgical interventions. However, it has not been studied for a group undergoing a single surgical procedure for KCOT to reduce the influence of confounding factors. Furthermore, little is known about any relationship between expression of cell proliferative markers and the recurrence in the KCOT in terms of recurrent risks for time dependent variables (18). The results of our study demonstrated higher expression of Ki-67 and AgNOR count in recurrent lesions comparing to non-recurrent lesion. On the other hand, Li et al, reported that no significant difference between simple (non-recurrent) and recurrent lesions regarding the expression of Ki-67 expression (35).

The limitation of this study is that only two markers have been evaluated. On the other hand, important point of this study is the demonstration of the positive correlation in between Ki-67 and AgNORs in suprabasal cell layers of KCOTs. This significant positive correlation was also reported by Gadball et al (6).

Another limitation is the low sample size of the recurrent lesions. In our study the recurrence was present only in 3 out of 22 subjects. Similarly, Kuroyanagi et al, (18) reported 4 recurrent lesions among 32 subjects diagnosed with KCOT. They reported that Ki-67 was found higher in the recurrent group and they suggested that this marker can be recommended as a prognostic factor. Our results are consisting with Kuroyanagi et al. (18) regarding the expression of Ki-67. Additionally, according to our results, we recommended that AgNOR might be useful as a prognostic marker in KCOTs.

Conclusion

Ki-67 and AgNOR might be useful as a prognostic marker in KCOTs. A standard protocol needed for the evaluation of AgNORs. The positive correlation of these markers reinforced the neoplastic character of the KCOT. The higher expression of these markers in recurrent lesions is important in order to consider additional surgical interventions to improve prognosis.

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Competing Interests

The authors have declared that no competing interest exists.

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