

Molecular biomarkers of cell proliferation in ameloblastomas

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Abstract

Cell proliferation is a vital biological process that is important for all living organisms because of its role in growth and the maintenance of tissue homeostasis. The control of this important process differs greatly among benign and malignant neoplasms, and the evaluation of cell proliferation in neoplasms has become a common tool used by pathologists to provide useful information pertaining to diagnosis, clinical behavior, and treatment. The usefulness of information regarding cell proliferation has led to numerous studies on the value of these methods for diagnosing different types of tumors and for clinical decision making. Ameloblastomas are no exception. This review discusses the use of several classical molecular proliferation markers, including Ki-67, proliferating cell nuclear antigen, cyclin D1 and DNA topoisomerase II alpha, to characterize ameloblastomas and proposes the use of new proliferation markers used previously to characterize other neoplasms. The use of these biomark-

ers offers valuable opportunities to evaluate the biological behavior of this type of odontogenic tumor.

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Key words: Ameloblastoma; Ki-67; Proliferating cell nuclear antigen; cyclin D1; DNA topoisomerase

Core tip: Specific molecular markers are characteristic of particular cellular events such as proliferation, and in this context, "proliferation markers" refer to specific proteins or other factors in actively growing and dividing cells, whose presence serves as an indicator for such cells. In this mini-review, we aim to provide an overview of the methods currently available for the assessment of proliferation, and we review the different cell proliferation markers used to assess the biological behavior of ameloblastomas. In addition, we propose a new marker.

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INTRODUCTION

New cells are generated from pre-existing cells through an ordered sequence of events that is repeated. These events constitute the cell cycle. Traditionally, the cell cycle is divided into stages, the duration of which varies depending on the cell type.

The division of an original cell into daughter cells requires prior DNA replication and the synthesis of various proteins associated with this replication step, as well as the production of structures and organelles for the new cells. These processes occur at the interphase of the cycle, which itself is divided into phases: G1 (Gap 1), S

(Synthesis) and G2 (Gap 2).

When all the conditions necessary for division are met, M stage (or division) starts, which results in the separation of the chromosomes and then the division of the cytoplasm, known as cytokinesis. Some cells can remain in a state of active metabolism for a very long time without replicating their DNA or dividing. These cells are in G0 phase or the quiescent state. G0 is also considered a post-mitotic state^[1].

The different cell types divide in a regulated manner. Certain environmental changes, such as temperature variations, changes in pH, nutrient scarcity and contact with neighboring cells, can slow down the cell cycle. Additionally, the presence of growth factors and hormones can trigger a series of intracellular processes that stimulate cell division. Cell proliferation can be defined as an increase in the number of cells as a result of cell growth and cell division.

In neoplastic processes, the abnormal and uncontrolled proliferation of cells is observed, and the cell cycle is altered. The assessment of cell proliferation activity in tumors has become a common tool used by histopathologists to provide useful information for assessing and predicting the behavior of tumors—that is, their likelihood of local recurrence, their metastatic potential, and the growth of metastases, and thus the likely duration of disease-free survival and survival to death^[2]. Today, the most common method for evaluating proliferative activity is the use of immunohistochemical techniques.

Immunohistochemical staining is widely used in the identification of abnormal cells such as those found in cancerous tumors. This technique is also widely used in basic research to understand the distribution and localization of biomarkers and differentially expressed proteins in different parts of a biological specimen.

Specific molecular markers are characteristic of particular cellular events such as proliferation, and in this context, “proliferation markers” refer to specific proteins or other factors in actively growing and dividing cells, whose presence serves as an indicator for such cells^[3].

Two requirements have been postulated for this type of marker: (1) the antigen should be continuously present during the cell cycle of all cell types; and (2) the transition to a nonproliferative state from any step of the cell cycle should be followed by a rapid disappearance of the antigen^[4].

Odontogenic tumors constitute a group of heterogeneous lesions that range from hamartomatous or non-neoplastic tissue proliferations to benign and malignant neoplasms with variable aggressiveness.

Odontoma is the most common odontogenic tumor, but it is considered a non-neoplastic lesion. Ameloblastoma is the most common odontogenic neoplasm. According to the 2005 Histological Classification of Tumors of the World Health Organization, ameloblastomas are divided into four variants: solid/multicystic, extrasosseous/peripheral, desmoplastic and unicystic. There exist several histological subtypes: follicular, plexiform, acanthomatous, granular and basal cell. Although ameloblastomas are classified as benign neoplasms, they can be locally invasive and destructive tumors of the jawbone. The molecular

mechanisms that regulate cell growth and invasion in ameloblastomas are unknown. Determining the proliferative activity of ameloblastomas may provide important information regarding the appropriate treatment strategy.

In this mini-review, we aim to provide an overview of the methods currently available for the assessment of proliferation, and we review the different cell proliferation markers used to assess the biological behavior of ameloblastomas.

There are many methods for determining the level of proliferative activity in different types of tumors, including the analysis of the mitotic index, flow cytometry, silver staining (AgNOR), and immunohistochemistry techniques. The last two are the most widely used techniques to study ameloblastomas.

It is important to clarify that there are more specific and sensitive techniques for determining the presence of these proliferation markers such proteomics techniques which allow to know what proteins are present or absent in these tumors. Another technique quantitative, sensitive and highly specific is the real-time polymerase chain reaction that allows determining the expression levels of genes in the ameloblastoma.

Both techniques are more expensive and more laborious than the immunohistochemistry technique that despite having less specificity and sensitivity has the advantage of being able to display “*in situ*” the presence of proteins, important data for understanding how the tumor proliferates.

MOLECULAR MARKERS

Silver binding nucleolar organizer region

Several methods have been used for the identification of proliferating cells in tissue sections with the aim of using them as markers of impending malignancy. One among of these methods is the silver binding nucleolar organizer region (AgNOR) technique.

Nucleolar organizer regions (NORs) are segments of DNA that are closely associated with nucleoli, which contain the ribosomal DNA. These regions therefore contribute strongly to the regulation of protein synthesis. NORs are argyrophilic and can therefore be visualized using a silver staining technique, what has led to the use of the term AgNOR^[2]. AgNOR staining is a simple one-step staining technique that overcomes the disadvantages of other techniques, such as the requirements for sophisticated equipment and technical expertise, high cost and long run-time^[3,5]. The amount of AgNOR protein, estimated during interphase, can be used as a marker of cell proliferation and has prognostic value for several human cancers.

In the study by Seifi *et al.*^[6], the number of AgNOR dots in solid/multicystic ameloblastomas was found to be higher than that in unicystic ameloblastomas.

Coleman *et al.*^[7] reported that unicystic ameloblastomas lined with characteristic epithelium had a significantly lower AgNOR count than solid ameloblastomas, residual dentigerous cysts and keratocystic odontogenic tumors, and these authors concluded that AgNOR counts are not

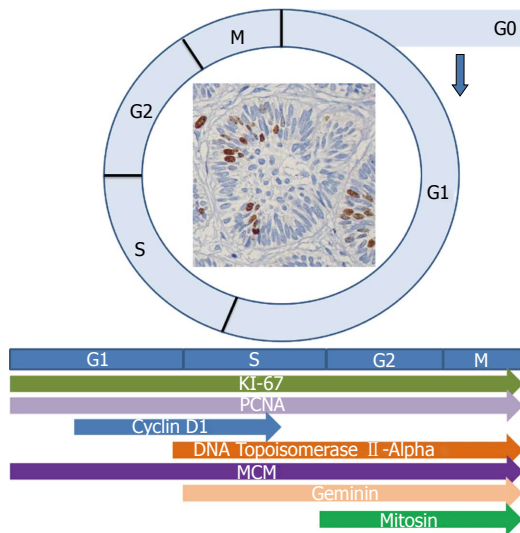


Figure 1 Presence of proliferation markers proteins during the cell cycle phases. The figure illustrates the presence of each marker of cell proliferation at different phases of the cell cycle. G1: Gap 1; G2: Gap 2; S: Synthesis; M: M-phase; PCNA: Proliferating cell nuclear antigen; MCM: Minichromosome maintenance complex.

of diagnostic significance and cannot be used to distinguish the various types of odontogenic cysts from one another or from unicystic ameloblastomas.

In terms of histological patterns, a significant difference has been found only between the follicular and plexiform types^[8]. A significantly higher number of Ag-NOR dots per nucleus was found in follicular ameloblastoma cells than in plexiform ameloblastoma cells^[9].

IMMUNOHISTOCHEMISTRY OF PROLIFERATION-ASSOCIATED ANTIGENS

Ki-67

The monoclonal antibody Ki-67 was first described in 1983 by Gerdes *et al.*^[10], who suggested that it might be used as a marker for proliferating cells. The Ki-67 antigen (Ki-67) is a classic marker of cellular proliferation and has been widely applied in the diagnostic, research and drug-discovery fields. The Ki-67 antigen was originally defined by the monoclonal antibody Ki-67, the name of which was derived from the city of origin (Kiel) and the number of the original clone in the 96-well plate^[10]. The expression of Ki-67 occurs during all phases of the cell cycle except the G0 phase and the early G1 phase (Figure 1), and the expression level increases as cell proliferation progresses, especially in the S phase, with peaks in the G2 and M phases. This protein is then degraded rapidly after mitosis^[3]. The standard antibody for the detection of Ki-67 is MIB-1. The fraction of MIB-1-positive tumor cells (the MIB-1/Ki-67 labeling index) is often correlated with the clinical course of the cancer, and Ki-67 is of prognostic value for many types of malignant tumors^[11]. There have been numerous studies that have aimed to determine the

Table 1 Cell proliferative activity measured using Ki-67 and/or proliferating cell nuclear antigen antibodies in ameloblastomas

Ref.	n	Type (n)	Ki-67	PCNA
Kim <i>et al.</i> ^[40]	38	Unicystic (13)		+
		Solid/Multicystic (25)		+
		Follicular		
		Plexiform		
		Acanthomatous		
		Granular		
		Basal Cell		
		Ameloblastic Carcinoma		+
Fonaoka <i>et al.</i> ^[41]	23	Plexiform (15)		++
		Follicular (5)		+++
		Unicystic (3)		+
Ong'uti <i>et al.</i> ^[18]	54	Plexiform (30)	+	
		Follicular (24)	++	
Piatelli <i>et al.</i> ^[42]	22	Unicystic (5)		+
		Solid/Multicystic (13)		
		Plexiform (5)		++++
		Follicular (4)		++
		Acanthomatous (4)		+++
Nagao <i>et al.</i> ^[43]	30	Plexiform (15)	++	
		Follicular (15)	+	
Sandra <i>et al.</i> ^[12]	32	Plexiform (9)	++++	++++
		Follicular (9)	++++	++++
		Acanthomatous (3)	+++	+++
		Basal Cell (3)	+++++	+++++
		Desmoplastic (3)	+	++
		Unicystic (5)	++	+
Han <i>et al.</i> ^[19]	70	Follicular (ND)	+++	
		Plexiform (ND)	++	
		Unicystic (ND)	+	
Meer <i>et al.</i> ^[15]	20	Solid/Multicystic (10)	+	+
		Unicystic (10)	++	++
Galvão <i>et al.</i> ^[44]	16	Follicular (7)		++++
		Plexiform (4)		++
		Acanthomatous (3)		+++
		Basal Cell (2)		+
Bologna-Molina <i>et al.</i> ^[3,13,45]	120	Solid/Multicystic (66)	+++	+++
	10	Unicystic (87)	++++	++++
	161	Peripheral (3)	++	++
		Desmoplastic (5)	+	+
		Ameloblastic Carcinoma (4)	+++++	+++++
Rizzardi <i>et al.</i> ^[14]	15	Peripheral (2)	+++	
		Unicystic (2)	++	
		Solid/Multicystic (11)	+	
Salehinejad ^[46]	30	Plexiform (15)		+
		Follicular (12)		+
		Acanthomatous (3)		++
Yoon <i>et al.</i> ^[47]	17	Ameloblastomas (10)	+	
		Ameloblastic Carcinoma (7)	++	
Maya <i>et al.</i> ^[21]	15	Plexiform		+++
		Follicular		++
		Unicystic		+

The table describes the immunohistochemical studies performed with markers proliferating cell nuclear antigen and Ki-67 from the year 1994 to date. PCNA: Proliferating cell nuclear antigen.

proliferative capacity of ameloblastomas using the Ki-67 marker (Table 1). However, the comparisons of solid or multicystic tumors with unicystic tumors have yielded conflicting results. Some authors found a higher rate of positivity for Ki-67 in the solid/multicystic type^[12], but other authors obtained different results, finding that the unicystic type had greater Ki-67 positivity^[13-15]. Given that

several clinicopathological studies have found that solid ameloblastomas are more aggressive than unicystic ameloblastomas^[16-19], the higher index of cell proliferation in unicystic ameloblastomas (determined using the Ki-67 antibody) found in some studies appears contradictory. This finding could be explained by the fact that unicystic ameloblastomas contain fewer stellate reticulum-like cells than solid/multicystic ameloblastomas, and consequently, most of the cells counted corresponded to basal or suprabasal layers, which are more likely to be positive. In other words, the proportions of the diverse types of epithelial cells, as well as the different mechanisms of growth in unicystic ameloblastoma and solid/multicystic ameloblastoma, may influence the results of the proliferation index^[13].

When histological subtypes were studied Ong'uti *et al.*^[18] in a study of 54 cases of ameloblastoma in Kenya, these researchers found that follicular ameloblastomas had a higher proliferation index than the plexiform variant. These results are similar to those of Han *et al.*^[19], who studied a Chinese population and found a slight predominance of a higher proliferation index in the follicular variant. Sandra and colleagues included the basal cell variant in their study, and this variant was found to have greater positivity for Ki-67 than the follicular variant^[12]. In our previous study, we found similar results, with the follicular variant having a higher proliferation index^[13].

Proliferating cell nuclear antigen

Proliferating cell nuclear antigen (PCNA) is a nuclear nonhistone protein that is necessary for DNA synthesis and is an accessory protein for DNA polymerase alpha, the expression of PCNA occurs during all phases of the cell cycle and the level of this protein is elevated during the G1/S phase of the cell cycle (Figure 1). PCNA expression can be used as a marker of cell proliferation because cells remain in the G1/S phase for a longer time when proliferating. Furthermore, this protein has an essential role in nucleic acid metabolism as a component of the DNA replication and repair machinery^[20,21].

Multiple studies using PCNA have been performed to determine the rates of cell proliferation in various types of ameloblastomas, but the results are contradictory (Table 1). Some authors did not find any relevant differences between the different types and subtypes of ameloblastomas^[3,11,22]. This result is most likely because PCNA is also involved in DNA repair. Because there is active ongoing DNA repair in many tumors, PCNA may also be upregulated in non-proliferating cells. Indeed, in some tumors, 100% of cells show positive staining. Therefore, after an initial period of popularity, PCNA is no longer considered a reliable proliferation marker in tumors^[2]. Despite this conclusion, there are still numerous studies using PCNA as the first-choice marker of cell proliferation in ameloblastomas (Table 1).

Cyclin D1

The cyclins, together with cyclin-dependent kinases (CDKs), are the proteins responsible for the orderly

progression of cells through the cell cycle. Cyclin D1 is amplified and/or overexpressed in a substantial proportion of different human tumors. Increased cyclin D1 expression occurs relatively early during tumorigenesis^[23]. Changes in the genes encoding these proteins as well as changes in the expression levels of these proteins are found during the process of carcinogenesis. The overexpression of this protein leads to uncontrolled cell proliferation and tumor development^[23]. Cyclin D1 is the regulatory subunit of the holoenzyme that phosphorylates and, together with sequential phosphorylation by cyclin E/CDK2, inactivates the cell-cycle inhibiting function of the retinoblastoma protein (pRb). pRb serves as a gatekeeper for the G1 phase, and passage through this restriction point leads to DNA synthesis. Thus, cyclin D1 promotes progression through the G1/S phase of the cell cycle (Figure 1)^[24,25].

Follicular and plexiform ameloblastomas express cyclin D1 in many peripheral columnar or cuboidal cells and in some central polyhedral cells. No distinct difference in the reaction was detected between these two main tumor types^[26]. Kumam *et al.*^[27] found that 19/25 follicular ameloblastomas were positive for staining with the cyclin D1 antibody, as were 9/10 plexiform ameloblastomas and 3/4 unicystic ameloblastomas.

DNA topoisomerase II alpha

DNA topoisomerases are enzymes that disentangle the topological problems that arise from double-stranded DNA. Many of these problems can be solved by generating either single- or double-strand breaks. However, when it is necessary to alter the DNA topology by introducing transient double-strand breaks, only DNA topoisomerase II (Top2) can fix the problem^[28]. Type II topoisomerases change the DNA topology by generating transient DNA double-strand breaks. The DNA topoisomerase II alpha (TOP2α) is one of the major nuclear proteins, with peak expression in the S to G2/M phase. It is involved in nearly every aspect of DNA metabolism, playing an important role in chromosome organization and segregation^[28].

Kumamoto *et al.*^[26] studied the presence of this protein in tooth germs and ameloblastomas, finding lower expression than that reported for Ki-67.

New cell proliferation markers

The usefulness of a marker for tumor diagnosis must be tested for each tumor type and application. Only those markers that have proven to be useful in practice should be considered. These three new cell proliferation markers have been studied in various types of cancer, although there are not currently any reports demonstrating their usefulness in ameloblastomas.

Geminin and minichromosome maintenance complex

Minichromosome maintenance complex (MCM2-7) and geminin have important roles in the prevention of DNA re-replication during the cell cycle. MCM proteins are

expressed cells in all phases of the cell cycle, including cells that exit the G0 and enter the G1 phase^[29]. Geminin is present from the G1/S transition to the early M phase. Thus, MCM is a G0/G1/S/G2/M-phase marker, and geminin is an S/G2/M-phase marker (Figure 1)^[30]. MCM proteins are known to contribute to the regulation of transcription, chromatin remodeling and checkpoint responses. The activated MCM complex appears to play a key role in DNA unwinding, acting as a DNA helicase^[31]. Following the initiation of DNA replication during the cell cycle, geminin inhibits the reloading of the MCM complex onto chromatin and prevents DNA re-replication during the same cell cycle^[32,33].

In recent years, these two proteins have been studied in various types of malignant neoplasms and have been shown to be very useful prognostic markers^[34,35].

Mitotin

Mitotin, also termed centromere protein F (CENP-F), is a member of the human centromeric protein family. This protein is associated with the centromere/kinetochore complex and is expressed in all active phases of the cell cycle, with a maximum in G2 and M phases^[36]. At the end of mitosis, CENP-F is rapidly proteolyzed by the proteasome. Accumulating evidence suggests that CENP-F is an important protein involved in chromosome alignment and kinetochore-microtubule interactions.

The cell cycle-specific expression of CENP-F makes it a potential marker of proliferation. Indeed, CENP-F is correlated with tumor proliferation in a variety of human tumors, including lung cancer^[37], non-Hodgkin lymphoma^[38] and salivary gland tumors^[39].

CONCLUSION

The evaluation of cell proliferation activity in tumors provides useful information related to diagnosis, clinical behavior, treatment and research.

Note that the use of these biomarkers alone are not useful for the diagnosis of ameloblastoma, the diagnosis is based on clinical and histopathologic features, but yet proliferation molecular biomarkers provide important information when predicting the prognosis of patients with ameloblastoma, so the histopathological types together with proliferation marker expression could be useful tools for evaluating the biological behavior of ameloblastomas.

Over the past several decades, various cell proliferation biomarkers have been demonstrated to be useful in the study of various types of neoplasms, and these markers have been studied in some odontogenic tumors and ameloblastomas. The Ki-67 protein remains an excellent operational marker for determining the growth fraction of a given cell population and is considered the gold standard method for the evaluation of proliferation activity.

Despite the abundance of research, the results regarding which type of ameloblastoma has the highest rate of cell proliferation remain controversial. One problem is the lack of standardization regarding how to determine

the cell count among many studies. In addition, ameloblastomas are polymorphic odontogenic tumors, with various types and variants, and the specific histomorphology of each type and the different mechanisms of growth may influence the observed proliferation index counting.

In recent years there have been found some new molecular biomarkers directly involved in the proliferation biology of tumors. Today new monoclonal antibodies are being tested in different tumors, hence the importance of new research using these new markers in ameloblastomas.

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