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SATB2 EXPRESSION IN MESENCHYMAL AND MIXED ODONTOGENIC TUMORS

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ABSTRACT

SATB2 Expression in Mesenchymal and Mixed Odontogenic Tumors.

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Odontogenic lesions are those originating from the tissues of the developing dentition and surrounding tissue, excluding osteogenic lesions. Often, these lesions histologically mimic other entities, sometimes even malignancies. While immunohistochemical markers are often utilized to tease overlapping lesions apart, a reliable odontogenic mesenchyme marker has not been identified at this time. SATB2, an osteogenic marker proven to be involved in odontogenesis, has been identified as a potential marker for odontogenic mesenchymal and mixed neoplasms. SATB2 immunoreactivity was assessed among odontogenic myxomas, central odontogenic fibromas, peripheral odontogenic fibromas, and ameloblastic fibromas/ameloblastic fibro-odontomas, and was scored using an H test (range 0-300). Staining with an H score ≥100 was deemed "strongly positive", and a lesion group was considered "strongly positive" if ≥80% of the included specimens showed strongly positive H scores. Limited preliminary data demonstrated strongly reactive SATB2 staining (≥80%) among the odontogenic mesenchymal lesions, with reduced (<80%) – but clinically relevant – staining observed within the peripheral odontogenic fibromas. Thus, SATB2 appears to be a useful marker for the identification and diagnosis of odontogenic mesenchymal and mixed neoplasms.

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LIST OF ABBREVIATONS

SATB2	Special AT-rich sequence-binding binding protein 2
MAR	Matrix-attachment regions
H&E	Hematoxylin and eosin
SAS	*SATB2*-associated syndrome
WRNMMC	Walter Reed National Military Medical Center (WRNMMC)
JPC	Joint Pathology Center
WHO	World Health Organization
COF	Central Odontogenic Fibroma
POF	Peripheral Odontogenic Fibroma
AF	Ameloblastic Fibroma
AFO	Ameloblastic Fibro-odontoma
RNA	Ribonucleic Acid
mins	minutes
PII	Personally Identifiable Information
µg	microgram
ml	milliliter
PI	Primary Investigator

CHAPTER 1: Introduction

ODONTOGENIC NEOPLASMS

Odontogenic lesions are those lesions originating from the tissues of the developing dentition and surrounding tissue, excluding the osteogenic lesions of the maxilla and mandible. As odontogenesis is specific to the mandible and maxilla, these lesions exclusively occur within the confines of the oral and maxillofacial region. Odontogenic lesions can be intraosseous or extraosseous, and may be associated with, in place of, or in proximity to a developing tooth. They can be categorized into two progenitor cell types – odontogenic epithelium or mesenchyme. Epithelial odontogenic lesions develop from odontogenic epithelium, which gives rise to the hard tissue structures of the tooth. Mesenchymal odontogenic lesions develop from the mesenchymal tissue of dental origin, which gives rise to the periodontal ligaments, dental follicle, and dental papillae. Lesions can be purely epithelial or mesenchymal, or mixed epithelial-mesenchymal.[1]

Odontogenic lesions can vary in presentation; they range from cysts to solid tumors, unilocular to multilocular, and radiopaque to radiolucent. Many odontogenic lesions share varying or overlapping histologic and radiographic features, particularly those arising from the odontogenic mesenchyme. For example, an ameloblastic fibroma histologically presents almost identically to a developing odontoma, but also shares the same ameloblastic epithelium as an ameloblastoma or an adenomatoid odontogenic tumor. Odontogenic mesenchymal and mixed neoplasms may even appear like non-odontogenic lesions. Consider the odontogenic fibroma. By definition, an odontogenic

fibroma is a mass composed of fibroblastic spindle cells that contains odontogenic rests; however, these rests do not need to be identified histologically for diagnosis, making this lesion easily confused for a fibroma, fibromatosis, or peripheral nerve sheath tumors. Odontogenic mesenchyme-derived lesions also have malignant mimics; an odontogenic myxoma may be mistaken for a myxoid chondrosarcoma and an odontogenic fibroma may be mistaken for a metastatic carcinoma or an odontogenic epithelium-derived carcinoma such as a sclerosing odontogenic carcinoma.

Given that the histologic and radiographic presentation is varied and histologic overlap is not uncommon between odontogenic lesions and their mimics, particularly those arising from the dental mesenchyme, a challenge is presented for pathologists, and histopathologic diagnosis of these lesions may be incorrect if a feature goes unnoticed. While it often is not difficult to identify a destructive malignant process or an aggressive benign entity (such as an ameloblastoma) once it has presented with features of destruction and clinical expansion, differentiation among the more indolent benign lesions and the occasional subtle early malignancy is where this challenge often exists. While in the case of benign entities, misdiagnosis often may not alter the patient treatment or prognosis, in cases such as the odontogenic myxoma, proper diagnosis of odontogenic lesions is necessary to guide more aggressive surgical treatment and watchful monitoring due to increased recurrence rates. It goes without saying that a misdiagnosis of a malignancy can have devastating consequences. Not only is the proper diagnosis of lesions clinically important, but studies on the progression and natural history of a lesion or changes in its demographic profile are also only as strong as the

diagnoses rendered; ensuring that they are accurate serves to strengthen the veracity of the epidemiologic findings as well.

SATB2

Special AT-rich sequence-binding binding protein 2 (SATB2) is a nuclear matrix protein that regulates osteoblast differentiation through interactions with the osteogenic transcriptional regulators *RUNX2* and *SP7/Osx*. SATB2 binds to nuclear matrix-attachment regions (MARs), organizing chromatin looping in order to permit appropriate expression of cell-type specific differentiation genes. Loss of SATB2 expression in mouse models results in skeletal hypoplasia and reduced osteogenic gene expression. Over-expression of *SATB2*, in contrast, promotes bone expression.[2,3] Alteration of *SATB2* in humans result in *SATB2*-associated syndrome (SAS), which is characterized by bowing tibias, low bone density and high fracture rate, osteoporosis, craniofacial abnormalities, developmental delays, and behavioral issues. Craniofacial features specific to SAS include delayed tooth eruption, cleft palate, large dentition, delayed root formation, delayed/missing second bicuspids, and dental malformations, such as taurodontism. Patients were also found to have high-arched palates leading to crowding/rotation of anterior dentition and loss of attached gingiva.[4]

SATB2 immunostain has been shown to function as an osteogenic marker, demonstrating nuclear expression in osteosarcomas as well as many other tumors involving osteoblastic differentiation such as osteoblastoma, fibrous dysplasia, osteoid osteoma, and bony elements of dedifferentiated chondrosarcomas and liposarcomas.[5] MicroRNA and messenger RNA analysis of dental mesenchymal stem cells further elucidated SATB2's function as a signature gene involved in regulation of

osteoblasts/osteogenesis.[6] Studies have also showed that SATB2 is involved in the differentiation of ameloblasts and odontoblasts, formation of dentin, and regulation of dental pulp functions. Additionally alterations in genes that involve the epithelial-mesenchymal interaction necessary for odontogenesis – in which SATB2 has been shown to play a role – can lead to a variety dental developmental abnormalities. A full *SATB2* knock-out in a murine model showing craniofacial abnormalities and hypodontia/anodontia supports this finding. Given these findings, *SATB2* mutations likely contribute to not only the craniofacial findings in SAS patients, but also the dental findings.[7]

While SATB2 has demonstrated a use in the immunohistochemical diagnosis of osteogenic lesions, little is known about its use as an immunohistochemical marker for odontogenic lesions. Given that SATB2 has been shown to be involved in odontogenesis and is present in the mesenchymal stem cells of teeth, one can postulate that odontogenic lesions, specifically of mesenchymal origin, may show positive staining with SATB2. We seek to classify the staining patterns of SATB2 in an attempt to develop a novel use of SATB2 as a marker for odontogenic mesenchymal lesions, improving the diagnosis and treatment of patients with histologically complicated specimens.

CHAPTER 2: Materials and methods

STUDY DESIGN

Using de-identified/randomized historic formalin-fixed, paraffin-embedded specimen blocks from the Joint Pathology Center (JPC) and Walter Reed National Military Medical Center (WRNMMC), IHC staining for SATB2 expression was assessed among odontogenic mesenchymal and mixed neoplasms (odontogenic myxoma, central odontogenic fibroma, peripheral odontogenic fibroma, and ameloblastic fibroma/fibro-odontoma) and compared against dental follicular tissue (positive control), and mucoceles (negative control) in an attempt to determine whether SATB2 is an effective immunostain for the identification of lesions of odontogenic mesenchyme origin.

STUDY PROCEDURES

Paraffin blocks were identified and coded (See Data collection section for acquisition procedures). The paraffin blocks were chilled on ice, and 4 μm sections were cut on microtomes by WRNMMC or JPC histology staff and placed on a charged glass slide, resulting in two unstained slides per block. One of each pair of generated unstained slides was stained with Hematoxylin and Eosin (H&E) at their respective sites. WRNMMC H&E slides were generated on the Roche Ventana HE600 System using the Routine Protocol as detailed in the Basic Operator Overview [100137.2904 (version 1.0)] and further clarified in the User Manual 1.9.5 (100137.2902). SATB2 staining of the remaining unstained slides was completed on Ventana Ultra stainers using the UltraView DAB kit. Slides were de-paraffinized and treated for 20 minutes (mins) at 99 C with Cell condition 1 (tris-based buffer) pH 8.0-8.5. SATB2 antibody (SATB2, clone EP281, REF#

384R-18 from Cell Marque received in pre-dilute form of 7 ML at 5.26µg/ml) was incubated for 48 mins at 37 C. Hematoxylin and Bluing was used as counterstain for 4 mins each. The JPC generated slides are pending approval and protocol generation and will be provided at a later date. They will be cut and coded by JPC histology technicians following the same steps as the WRNMMC-generated slides. The JPC laboratory will perform the H&E staining on one of the two generated unstained slides per block. The H&E and unstained slides generated by the JPC will be sent to WRNMMC via a pre-existing courier service, WRNMMC will perform the SATB2 immunostaining as listed in this protocol, and the JPC-generated blocks will be combined with the WRNMMC specimens.

Slides were assessed for strength and breadth of positive SATB2 nuclear immunoreactivity among tumor cells. Corresponding H&E slides were utilized to confirm that the recorded staining is limited to only the tumoral cells. In order to account for the inherent variation of staining intensity that can occur in any histologic specimen, the H score protocol of Xu et al was utilized.[8] The varying intensities (0 = no reaction, 1+ = mild reaction, 2+ = moderate reaction, 3+ = intense reaction) of positive nuclear staining within the tumor cells and the associated percentage of positive staining tumor cell nuclei at each intensity was recorded. These values were multiplied, and a sum of the staining populations was acquired using the following formula:

(3 × percentage of strongly positive nuclei + 2 × percentage of moderately positive nuclei + percentage of weakly positive nuclei)

Or

Σ ($T_i \times T_{\%}$), where T_i = staining intensity (range: 0-3) and $T_{\%}$ = percentage of nuclei demonstrating associated T_i (0-100%)

The above calculation resulted in a range of 0-300. Values ≥100 were considered diffuse and strongly positive, and recorded as "positive", while values <100 were defined as "negative".[8]

Since interpreting immunostaining is challenging, as no two stains behave alike, and some have stricter interpretation criteria than others[9], a strict qualifying criteria was defined. Sensitive immunohistochemical utility of SATB2 for any lesion was defined as ≥80% "positive" staining within the lesion subgroup (including positive control), with "negative" results within ≥80% of the negative control subgroup. A generalized immunohistochemical utility of SATB2 for all mesenchyme-derived odontogenic lesions was defined as ≥80% "positive" staining within ≥80% of *all* of the studied lesions.

DATA COLLECTION

Paraffin-embedded, formalin-fixed historical specimen blocks were identified from the repositories of both WRNMMC and the JPC. WRMNNC specimens were identified using a natural language search within the CoPath database using the following search terms: "central odontogenic", "odontogenic fibroma", "ameloblastic", "odontogenic myxoma", "mucocele", and "dental follicle". JPC specimens were identified using a repository search form for the same terms. Ten (10) specimens of each

of the following World Health Organization (WHO)-classified odontogenic mesenchyme-derived entities were identified: *odontogenic myxoma, central odontogenic fibroma, ameloblastic fibroma/fibro-odontoma, and peripheral odontogenic fibroma*.[10] Ten (10) specimens of dental follicular tissue were be used as a positive control, and ten (10) specimens of mucoceles were be used as a negative control. Each lesion group (i.e. odontogenic myxoma, odontogenic fibroma, etc.) was randomly labeled A-F, and each specimen block was randomly labeled 1-10 by a third party histology technician, in order to ensure the reviewers were adequately blinded and Personally Identifiable Information (PII) was removed. Once the alphanumeric code was generated, one (1) H&E slide and one (1) SATB2 IHC slide was generated per specimen (see above for procedure) and provided to the Principal Investigator. The SATB2 nuclear staining of the tumor cells was categorized as "strong", "moderate", or "weak", and an H score was calculated (Appendix 1).

The slides will ultimately be reviewed by two additional board-certified oral & maxillofacial pathologists, where all three reviewers will individually score the staining patterns of each specimen and record their results and H scores (Appendices 2-3). An average H score for each lesion will then be generated (Appendix 4), which will be used to calculate the percentage (i.e. #/10, #%) of strongly positive (H score $\geq$100) lesions within *each* lesional group (COF/POF, AF/AFO, etc.) as well as an overall percentage (i.e. #/3, #%) of strong staining of *all* odontogenic lesions (excluding positive and negative controls) (Table 1).

DATA ANALYSIS

A descriptive table showing the means and confidence intervals (or medians with interquartile ranges, as appropriate) for H scores for each of the tumor types (including the positive and negative control sections) will be created (Appendix 4). In order to address whether H scores differ between tumor types after SATB2 staining is interpreted by pathologists, a mixed effects model will be performed using each H score as the dependent measure and tumor type as a fixed effect. A random effect of pathologist and section will be included to account for repeated measurements across samples. Individual pairwise differences between tumor types will be assessed using multiplicity corrected comparisons.

For descriptive purposes and to aid clinical interpretation, an H score of 100 will be used as the clinical interpretation cut-off value for an analysis of the number of each type of slides that are positive and negative using the **SATB2 staining method**. Each individual pathologist read for each slide will be assessed using this threshold, and binomial confidence intervals (using the Agresti & Coull (1998)) method will be reported for each tumor type (including the positive and negative control). Intraclass correlation coefficients and/or kappa statistics will be run on the H scores for each tumor type generated by each pathologist to evaluate the similarity/resemblance between pathologist interpretations of SATB2 staining. This will tell the degree of consistency of readings from the three reviewing pathologists.

CHAPTER 3: Results

As material sharing agreements are still underway, the data analyzed was only of those blocks generated by WRNMMC. Less than 10 specimens of several lesion groups (COF, AF/AFO, and Myxoma) could be acquired from the WRNMMC repository. As central and peripheral odontogenic fibromas are variants of one another, they were joined together into one group to meet the 10-specimen criteria. Following the initial generation of data, another central odontogenic fibroma block was discovered in the WRNMMC archives and was included in the COF/POF group, creating a sample size of 11.

Control tissues were assessed as an initial quality control measure, and the data analysis at this time was only performed by the Primary Investigator (PI)(Appendix 1), with the additional two reviewers' data to be collected and analysis completed (Table 1, Appendices 2-4) following acquisition of all remaining specimens.

CHAPTER 4: Discussion

SATB2 is a nuclear matrix protein known to regulate osteoblastic differentiation via interactions with RUNX2[2], and which is currently used as an immunohistochemical marker for colorectal carcinomas and osteosarcoma. Studies elucidating SATB2's role in odontogenesis indicated that it may demonstrate additional staining within odontogenic lesions, particularly of mesenchymal origin. In order to adequately assess the staining of SATB2 within odontogenic mesenchymal neoplasms, an H test was performed. This test was chosen because it doesn't just record the percentage of nuclei demonstrating positive staining; it further categorizes the staining into weighted categories of intensities (the stronger the intensity, the more weight the percentage value is given), which is more representative of the natural variations that occur within a specimen. This prevents the staining intensity value from being over- or underrepresented.

Initial assessment of the control tissue was adequate. The mucocele group showed no strong staining, fitting the criteria for a negative control, and the dental follicle group showed 8/10 (80%) strong staining. Ideally, the positive control should be entirely strongly stained; the decreased scores seen in some of the dental follicle specimens could be due to a non-representative biopsy of connective tissue adjacent to the follicular tissue, whose otherwise non-specific diagnosis was able to be specified due to the unique radiographic findings seen with hyperplastic dental follicles.

Appendix 1 shows that a significant portion of the assessed odontogenic lesions have strong staining as demonstrated by scores of ≥100 shown in ≥80% of most lesion groups, with the exception of the COF/POF group. Some of the values are close to a score of 100 and may meet our criteria once averaged with the other reviewers (Appendix

4), which could assist the COF/POF group in meeting our ≥80% criteria. Nevertheless, at this time the data shows that only 8/11 (73%) of the COF/POF group showed positive staining. Recall, however, that these two lesion groups were combined due to limited available specimens. Specimens A1-A4 and A11 represent the central odontogenic fibromas, and A5-10 represent the peripheral odontogenic fibromas; if they are counted independently, 4/5 (80%) of the COF lesions and 4/6 (67%) of the POF lesions are strongly positive. If we consider this, the only isolated lesion group currently not meeting our ≥80% criteria is the POF group. This is not surprising, as POFs are commonly mistaken for other benign gingival neoplasms; it's not unlikely that these low staining lesions were actually a mimic, such as a myxoid fibroma or neurofibroma.

A drawback to our ≥80% cutoff interpretation is that at this time, 80% of a sample size of 5 is not a significant value and could easily vary following the addition of more samples. This value was assigned simply as a way of ensuring that the lesions were strongly positive and abundantly sensitive for the sake of initial characterization, not because it is a validated criteria. In reality, the College of American Pathologists published a 2014 review of IHC interpretation guidelines, which defined "positive" nuclear staining as staining of any amount/intensity that is present in >5% of tumor cells[9]. Following these guidelines, we see that SATB2 becomes extremely sensitive for odontogenic mesenchymal lesions. The specificity of the stain suffers if this criteria is used, however, because some of the negative control staining would be considered sufficient. However, much of the recorded staining likely was non-specific or cytoplasmic staining and simply misinterpreted by the less-seasoned PI.

Additional limitations with the current study include the inherent variability and bias that exists in the study design – along with the fact that the biopsy may not adequately represent the diagnosis (as mentioned above) or could be entirely incorrect (as in the case of the POF specimens), the reviewers are humans and thus are providing a subjective staining interpretation, the sample sizes are low, and the staining itself - even in the external control tissue - was admittedly underwhelming, leading to lower staining scores.

CHAPTER 5: Conclusions

Odontogenic neoplasms are those which are derived from the cells involved in tooth development, the oral epithelium and mesenchyme. Whereas lesions derived from the oral epithelium are frequently easily-identified, lesions of the oral mesenchyme - to include mixed epithelial-mesenchymal lesions - show a great deal of histopathologic overlap not only with one another but with various other lesions, including benign, aggressive benign, and malignant entities. Until now, no immunohistochemical markers for lesions derived from the odontogenic mesenchyme have been characterized. We believe that SATB2 has demonstrated significant sensitivity for lesions of odontogenic mesenchyme, and could serve as a useful diagnostic adjunct, especially for those pathologists who are unsure of the tumor origin or are simply unfamiliar with oral pathology and do not have access to a resident oral pathologist. That being said, caution should be taken when using SATB2, since it does stain a wide variety of cell types and could be easily misinterpreted. Our hope is that our remaining data supports our claims and will allow for larger studies to be performed moving forward.

Table 1. Percentage of strongly positive lesions per group and overall

	% strongly stained
A – COF/POF	(#/11), #%
B – AF/AFO	(#/10), #%
C - Myxoma	(#/10), #%
% of Above Lesion Groups with ≥80% Strong Staining	(#/3), #%

APPENDIX A

Appendix 1. Preliminary staining intensity and H score – PI

	% strongly stained	% moderately stained	% weakly stained	H score
A (Central and Peripheral Odontogenic Fibroma)				
1	0	50	25	125
2	35	25	5	160
3	30	15	5	125
4	10	25	10	90
5	25	20	15	130
6	10	45	0	120
7	0	0	15	15
8	2	15	50	86
9	75	0	0	225
10	55	15	5	200
11	5	40	10	11
B (Ameloblastic Fibroma/Fibro-Odontoma)				
1	60	20	5	225
2	15	35	15	130
3	35	10	25	150
4	20	35	20	150
5	75	5	0	235
C (Myxoma)				
1	50	25	5	205
2	10	25	35	115
3	0	25	55	105
4	5	65	0	145
5	0	0	10	10
6	3	60	5	134

	% strongly stained	% moderately stained	% weakly stained	H score
D (Dental Follicle, Positive Control)				
1	50	0	0	150
2	20	30	15	135
3	25	20	10	125
4	5	0	50	65
5	25	45	0	165
6	25	15	15	120
7	50	25	0	200
8	75	10	0	245
9	0	40	15	95
10	15	50	0	145
E (Mucocele, Negative Control)				
1	0	0	5	5
2	0	0	25	25
3	0	0	35	35
4	0	0	5	5
5	0	0	0	0
6	0	0	0	0
7	0	0	0	0
8	0	0	0	0
9	0	0	2	2
10	0	0	5	5

Appendix 2. Staining intensity and calculated H score – Reviewer #2

	% strongly stained	% moderately stained	% weakly stained	H score
A (Central and Peripheral Odontogenic Fibroma)				
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
Pending.				

Appendix 3. Staining intensity and calculated H score – Reviewer #3

	% strongly stained	% moderately stained	% weakly stained	H score
A (Central and Peripheral Odontogenic Fibroma)				
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
Pending.				

Appendix 4. Average H scores of all reviewers.

	H score
A (Central and Peripheral Odontogenic Fibroma)	
1	
2	
3	
4	
5	
6	
7	
8	
9	
10	
Pending.	

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