THE DETERMINATION OF AGREEMENT BETWEEN TRANSDUCER-LIKE ENHANCER SPLIT-1 (TLE-1) AND FLUORESCENCE IN SITU HYBRIDIZATION (FISH) IN SYNOVIAL SARCOMA CASES

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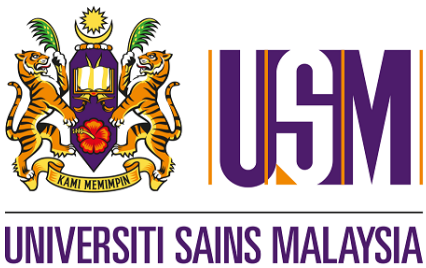
(ANATOMICAL PATHOLOGY)



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ABSTRAK

PERSETUJUAN ANTARA ‘TLE-1’ DENGAN ‘FISH’ BAGI KES ‘SYNOVIAL SARCOMA’

Kemunculan ‘Transducer-like Enhancer Split 1’ (TLE-1) sebagai satu penanda untuk ‘Synovial sarcoma’ kini telah memberi alternatif baru kepada pakar Patologi dalam mencapai diagnosa penyakit dan membezakan ‘Synovial sarcoma’ dengan kanser lain yang mempunyai bentuk histologi yang menyerupainya. Tujuan utama kajian ini adalah untuk menentukan persetujuan antara ekspresi TLE-1 dengan translokasi X;18 melalui ‘Fluorescence in Situ Hybridization’ (FISH). Namun, penghasilan tanda translokasi tidak berjaya dihasilkan melalui FISH, dan kajian ini telah menganalisis beberapa faktor penyebab kegagalan dan penyelesaian kepada teknik FISH.

Kajian ‘cross sectional’ telah dijalankan ke atas 27 kes, dengan mengkaji blok tisunya, yang mana kes-kes tersebut telah didiagnosikan sebagai ‘synovial sarcoma’ dari tahun 1999 hingga bulan Julai 2017. Pengkajian tentang ciri-ciri histologi telah dijalankan bagi mengenalpasti pegkelasan histologi ‘Synovial sarcoma’. Semua sampel tisu yang dipilih untuk diuji dengan ujian imunohistokimia TLE-1. Di samping itu, ujian FISH dijalankan keatas blok tisu menggunakan ‘prob’ SYT, di mana ia akan menghibridkan gen yang difokuskan. Penyiasatan terhadap punca kegagalan FISH dalam mendapatkan tanda gen yang diingini telah dijalankan.

Secara keseluruhannya, melalui analisis immunohistokimia, sebanyak 74.1% (20 kes) ‘synovial sarcoma’ menunjukkan tindakbalas positif ke atas TLE-1.Tindakbalas nuclear yang kuat (3+) adalah sebanyak 48.1% dan tindakbalas sederhana (2+) adalah sebanyak

25%. Sebanyak tujuh kes (25.9%) menunjukkan tindakbalas negatif ke atas TLE-1 (0 atau 1+). Kes yang tiada pewarnaan nucleus (0) adalah sebanyak 18.5% manakala tindakbalas lemah (1+) adalah sebanyak 7.4%). Tindakbalas TLE-1 tidak menunjukkan hubungan statistik yang signifikan dengan bentuk histologi 'Synovial sarcoma'. Disebabkan ketiadaan tanda translokasi X;18 diperolehi, pelan penambahbaikan telah diselidik seperti pre-pemanasan, penghancuran enzim, dan langkah-langkah hibridasi, yang mana telah diketahui umumnya sangat sensitive terhadap perubahan suhu, masa dah skala pH.

Kesimpulannya, TLE-1 adalah petanda immunohistokimia yang sangat berguna dalam hal mendiagnosi 'Synovial sarcoma' dan membezakan ia daripada kanser lain yang menyerupainya. Walaubagaimanapun, diakhirnya pendiagnosian 'Synovial sarcoma' hanyalah bergantung kepada pemerhatian dan penelitian pakar patologi dan dibantu dengan keputusan beberapa immunohistokimia. Pengetahuan tentang persetujuan antara TLE-1 immunohistokimia dengan ujian utamanya iaitu FISH diharapkan dapat dijadikan sebagai batu loncatan untuk tidak menjalankan ujian FISH, yang mana diketahui umum akan kesulitannya dan memerlukan kos yang tinggi. Namun, akibat daripada tiada tanda translokasi berjaya dihasilkan melalui FISH, kami tidak dapat mencari persetujuan tersebut.

ABSTRACT

THE DETERMINATION OF AGREEMENT BETWEEN TLE-1 AND FISH IN SYNOVIAL SARCOMA CASES

The emergence of transducer-like enhancer of split 1 (TLE-1) as a new immunohistochemical (IHC) marker for synovial sarcoma (SS) have recently offered an alternative diagnostic strategy to pathologists in differentiating SS from other histologic mimics. Our major aim is to determine the agreement between TLE-1 IHC expression with the translocation X;18 in fluorescence in situ hybridization (FISH) for diagnosing SS. However, due to poor t(X;18) FISH signal, this paper describes troubleshooting plans for FISH analysis that were carried out in determining positive signals for t(X;18).

We conducted a cross sectional study using 27 archived formalin-fixed paraffin embedded tissue blocks of synovial sarcoma, which was diagnosed in Hospital Universiti Sains Malaysia from year 1999 to July 2017. Histology assesment was performed to identify SS morphology subtypes. All samples were stained for TLE-1 by immunohistochemistry (IHC) and correlate morphology subtypes. In addition, (FISH) study were performed on formalin-fixed paraffin embedded tissue sections using break-apart SYT probe ,which hybridized to target the breakpoint gene. Troubleshooting for FISH were carried out in obtaining positive t(X;18) signal in SS cells.

From IHC analysis, 74.1% (20 cases) of synovial sarcoma showed positive nuclear immunoreactivity to TLE-1. Strong nuclear immunoreactivity (3+) was 48.% and moderate nuclear immunoreactivity (2+) was 25.9%. Seven cases (25.9%) were

negative to TLE-1 (score 0 or 1+). The cases with no nuclear staining (0) was 18.5% and weak nuclear immunoreactivity (1+) was 7.4%.

TLE-1 expression was not statistically significant with tumour morphology subtypes. Due to poor t(X;18) FISH signal, several troubleshooting plans were carried out i.e. pretreatment step, enzyme digestion and hybridization step, which the steps are known to be very sensitive to temperature, time and pH.

TLE-1 is a useful marker in diagnosing SS and to distinguish from its histological mimickers. The final diagnosis of SS is only by pathologist eyes, as we still rely on morphology and IHC interpretation. The presence of agreement between TLE-1 IHC and its gold standard test (FISH) is a ticket for not to proceed with the later, which is more laborious and expensive. However, failure of signal detection due to technical and wrong methodology, we unable to proof the agreement.

CHAPTER 1

1 INTRODUCTION

1.1 OVERVIEW OF SOFT TISSUE SARCOMA

Sarcomas are a group of rare solid tumours. They are arising from mesenchymal or connective tissue. Sarcomas account for about 1% of all adult malignancies and relatively uncommon group of malignancies. Among the family of mesenchymal malignancies, soft tissue sarcomas are the most common tumours, in which 80% of all sarcomas arise from soft tissue (Schöffski *et al.*, 2014). The WHO classification (4th Edition 2013) of soft tissue tumours incorporates detailed clinical, histological and molecular data. The usual approach to soft tissue tumour classification is by presumed cell lineage.

Soft tissue sarcoma is a very heterogenous. It can occur in all age group and all anatomical sites (Dangoor *et al.*, 2016). Majority of cases have unknown aetiology and some of them have certain genetic associations. Due to the heterogeneous sites of origin, it is difficult to clearly define the clinical features of the disease which leads to delay in getting the definitive diagnosis. They are often life threatening and cause therapeutic and prognostic challenges (Fletcher *et al.*, 1999). Apart from morphological features, there are multiple panels of immunohistochemistry available that can be used as a guide to diagnose soft tissue sarcoma. However, the variety and heterogeneity component of the sarcoma lead to diagnostic challenge among the pathologist. Some of the immunohistochemical markers proved to be more useful in clinical practice than others. However, it is generally appreciated that significant overlap in staining patterns can be seen in different tumour types. Some of the sarcoma share similar biology or can

be explained by known biologic mechanism (Banerjee *et al.*, 2013; Lin and Doyle, 2015). .

Rapid advance in molecular studies of soft tissue sarcomas help the pathologist to identify the cytogenetic aberration involved, particularly in a case of sarcoma of uncertain differentiation. These cytogenetic aberrations are frequently in the form of chromosomal translocation (Schaefer and Fletcher, 2018). Synovial sarcoma is one of the soft tissue sarcoma which falls in this category.

1.2 SYNOVIAL SARCOMA

Synovial sarcoma is a rare and aggressive soft tissue tumour. It is a mesenchymal spindle cell tumour, which display variable epithelial differentiation, including glandular formation. It is a translocation-associated mesenchymal neoplasm that represents around 10% of all soft tissue sarcomas (Foo *et al.*, 2011). A chromosomal translocation, t(X;18)(p11;q11) is specific for synovial sarcoma. The t(X;18) translocation most commonly fuses either the SSX1 or SSX2 gene on chromosome X to the SYT gene on chromosome 18. This result in the production of an SYT-SSX fusion protein (Minami *et al.*, 2014). In WHO classification (4th Edition 2013) synovial sarcoma is classified under group of tumour of uncertain differentiation.

From Malaysian National Cancer Registry Report 2007-2011, synovial sarcoma that arised from soft tissue and bone are 3.6% and 1%, respectively. Although synovial sarcoma initially believed to be of synovial cell in origin, the term is somewhat misleading. Synovial sarcoma is also encountered in areas with no apparent relation to synovial structures (Zulkarnaen *et al.*, 2012). .

1.3 CLINICOPATHOLOGICAL FEATURES – SYNOVIAL SARCOMA

Synovial sarcoma can affect all ages, but most prevalent in adolescents and young adults, age between 15 to 40 years of age. It occurs predominantly in male (Villaroel-Salinas *et al.*, 2012) . However, synovial sarcoma may occur at any age and equally distributed between sexes. SYT-SSX1 fusion transcript has a higher prevalence in males , and it is independently associated with increased risk of early distant recurrence (Tarkan *et al.*, 2014). This tumour is common seen in the deep soft tissue of the extremities, typically occur in limbs (Chuang *et al.*, 2013). From Norhamdan *et al.* (2008), synovial sarcoma is most commonly found in the head and neck, retroperitoneum and extremities. When they arise in the extremities, they are often located proximal to the hip or shoulder region. Synovial sarcoma is rarely presents as a primary bone tumour. If the tumour involves the surrounding soft tissue of the extremities, the underlying bone tend to be uninvolved (Zulkarnaen *et al.*, 2012).

Synovial sarcomas are frequently misdiagnosed as benign processes such as myositis, synovitis, haematoma, tendinitis or bursitis. Smaller lesions can be well-circumscribed and homogeneous .It can be mistaken for a benign mass and cause diagnostic pitfall. When the tumour is arising from unexpected site, it will lead to diagnostic challenges (Cheng *et al.*, 2012).

Synovial sarcoma is a slow-growing, high-grade malignant neoplasm with extensive metastatic potential. This pattern of slow tumor growth and the apparent harmlessness of symptoms often lead to late referral to a tertiary referral center. Consequently, the diagnosis and therapy are delayed (Krieg *et al.*, 2011). Patient with synovial sarcoma usually presented with either painful or painless mass. On gross section, the tumours are yellow to gray-white, well circumscribed and soft in consistency, which can be misinterpret as benign. The gross morphology of synovial

sarcoma is typically 3 to 10 cm in diameter.It also can grow up to 15 cm. Lesion size less than 1 cm have been reported. The less differentiated variants often grow more rapid and tend to be poorly circumscribed, with multiple areas of haemorrhage, variegated surface , necrosis and cystic formation (Siegel *et al.*, 2007).

Histologically, synovial sarcoma is composed of spindle cells with or without variable epithelial differentiation, including glandular formation. Due to its variable morphology, synovial sarcoma can be further classified as biphasic, monophasic and poorly differentiated. Most common type is monophasic, account for about 70% of cases. Biphasic synovial sarcoma has both epithelial and spindle cell components. Poorly differentiated synovial sarcoma is composed of sheets of atypical small blue cells. This is often misdiagnosed as primitive neuroectodermal tumour (Goldblum, 2014).

The epithelial component form glands with lumina. The glands are lined by epithelial cells with ovoid nuclei and abundant cytoplasm. Some of the epithelial cells contain mucin. Many tumours show prominent haemangiopericytomatous vascular pattern. The stromal collagen is usually wiry and have foci of dense fibrosis. Myxoid change can be present. Monophasic synovial sarcoma must be differentiated from all spindle cell tumours, including spindle cell carcinoma, hemangioperycytoma or malignant schwannoma.

Synovial sarcoma is a high grade sarcoma, which is characterised by local invasiveness and a tendency to metastasize early (Norhamdan *et al.*, 2008). The diagnosis of synovial sarcoma can be a challenging task, particularly on small biopsy specimens, as the morphologic features of this tumor can be mimicked by a variety of other neoplasms (Keith *et al.*, 2013). Synovial sarcoma has variable prognosis and

prognostic determinants are tumour size, stage at presentation, histology subtypes, stage at presentation, and age.

Thorough sampling is very important and must include the entire lesion. Histomorphology diagnosis remain important. The usage of multiple panels of immunohistochemical (IHC) marker are very helpful to differentiate synovial sarcoma from its mimickers. The IHC markers that are frequently used are CK AE1 & AE3, CK 7, EMA, CD34, BCL-2 and CD 99 (Banerjee *et al.*, 2013). The molecular study such as fluorescence in situ hybridization (FISH) or polymerase chain reaction (PCR) remain gold standard (Surace *et al.*, 2004; Yasuhiro *et al.*, 2012). However, the molecular study is not widely available in Malaysia due to financial limitation and lack of experienced personnel. Therefore, the production of immunohistochemical marker, which is more sensitive and specific is important.

1.4 IMMUNOHISTOCHEMICAL MARKER - TRANSDUCER –LIKE ENHANCER OF SPLIT 1 (TLE-1)

Transducer-like enhancer of split 1 (TLE1) has been recognized as a potential immunohistochemical marker for diagnosing a synovial sarcoma. It is an excellent biomarker for distinguishing synovial sarcomas from other soft tissue tumors or its histologic mimickers. Due to its higher sensitivity and specificity, it perform better than other currently used immunohistochemical markers (Seo *et al.*, 2011).

TLE-1 have been found in gene expression profiling, to be significantly overexpressed in synovial sarcomas (Foo *et al.*, 2011). It is a transcriptional co-repressors protein that inhibit Wnt signaling and other cell fate determination signals. From Jagdis *et al.* (2009), TLE1 was highly sensitive (86-97%) and specific (96- 100%) for synovial sarcoma in comparison to other currently available immunohistochemical

markers. From Knösel *et al.* (2010), 96% of synovial sarcoma cases showed positive TLE-1 strong to moderate staining.

There are four types of TLE genes, which are TLE1, TLE2, TLE3 and TLE4. All TLE family of genes, except TLE2 are expressed differently in synovial sarcoma. TLE proteins are temporarily expressed in embryogenesis, where they are involved in developmental processes such as neurogenesis, body patterning and hematopoiesis. TLE1 proteins are involved in the Wnt/β-catenin signaling pathway, which has been found to be good discriminator of synovial sarcoma (Jagdis *et al.*, 2009). TLE functions to repress genes involved in differentiation, and maintain the relatively undifferentiated histopathologic state seen in synovial sarcoma (Terry *et al.*, 2007). A carefully selected IHC panel will guide us in differential diagnosis, but does not always yield a definitive diagnosis. Thus, the ancillary study such as FISH is necessary to demonstrate the specific chromosomal translocation and thereby confirm the diagnosis.

1.5 MOLECULAR TEST - FLUORESCENCE IN SITU HYBRIDIZATION (FISH)

The introduction of molecular and cytogenetic studies have supplemented histology and immunohistochemistry test. The molecular study also gives better understanding of the biology of the synovial sarcoma. The translocation t(X;18)(p11.2;q11.2) is the cytogenetic hallmark of synovial sarcoma, in about 90 to 95% of cases (Minami *et al.*, 2014). The translocation t(X;18)(p11.2;q11.2) is not found in the histologic mimics, such as Malignant Peripheral Nerve Sheath Tumour (MPNST), Haemangiopericytoma, Leiomyosarcoma or Mesothelioma. In this study, one of the objective is to determine the incidence of t(X;18) in synovial sarcoma cases. However, this objective was not achieved as failure to get the signal translocation by FISH.

The fusion transcripts (SS18-SSX1 and SS18-SSX2) are detected by Fluorescence in situ Hybridization (FISH). This molecular test is very useful in certain cases of diagnostic dilemmas, such as cases occurring at unexpected sites, or when immunohistochemical profile is inconclusive for diagnosis (Chuang *et al.*, 2013). Thus, Fluorescence in situ Hybridization remain the gold standard test in view of its high sensitivity and specificity. From Arumugam *et al.* (2016) the sensitivity and specificity of FISH for known synovial sarcoma was 96.7%, and 100% respectively. There was no reported study about sensitivity or specificity of FISH in detecting subtypes of synovial sarcoma. The ability in detecting SYT-SSX fusion transcript allow us to determine the histological subtypes and its probable prognosis.

FISH able to determine the chromosomal translocation using SYT probe. The two forms of SYT-SSX fusion transcript, which are SYT-SSX1 and SYT-SSX2 , can be identified using RT-PCR. Kawai *et al.* (1998) found a significant correlation between SYT-SSX gene and histologic subtype of tumors, where the tumour containing SYT-SSX1 are biphasic and SYT-SSX2 are monophasic subtypes. These fusion transcripts appear to influence the histologic subtypes. SYT-SSX1 has the ability to induce architectural epithelial differentiation such as glandular formation (Kawai *et al.*, 1998).

CHAPTER 2

2.0 OBJECTIVES

2.1 General Objective

To determine the agreement between TLE-1 immunohistochemistry (IHC) marker and FISH analysis in the diagnosis of Synovial sarcoma.

2.2 Specific Objective

1. To identify the clinicopathological features of Synovial Sarcoma cases.
2. To describe the FISH method, in the detection of Translocation X;18, in the diagnosis of Synovial sarcoma.
3. To determine the agreement between TLE-1 immunohistochemistry (IHC) marker and FISH analysis in the diagnosis of Synovial sarcoma.

CHAPTER 3

3.0 MANUSCRIPT

3.1 Title:

Determining agreement between Transducer-Like Enhancer Spit-1(TLE-1) marker and FISH in synovial sarcoma cases.

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Competing interests: None

3.2 ABSTRACT

Background: The emergence of transducer-like enhancer of split 1 (TLE-1) as a new immunohistochemical (IHC) marker for synovial sarcoma (SS) have recently offered an alternative diagnostic strategy to pathologists in differentiating SS from other histologic mimics. Our major aim is to determine the agreement between TLE-1 IHC expression with the translocation X;18 in fluorescence in situ hybridization (FISH) for diagnosing SS. However, due to poor t(X;18) FISH signal, this paper describes troubleshooting plans for FISH analysis that were carried out in determining positive signals for t(X;18).

Methodology : We conducted a cross sectional study using 27 archived formalin-fixed paraffin embedded tissue blocks of synovial sarcoma, which was diagnosed in Hospital Universiti Sains Malaysia from year 1999 to July 2017. Histology assesment was performed to identify SS morphology subtypes. All samples were stained for TLE-1 by immunohistochemistry (IHC) and correlate morphology subtypes. In addition, (FISH) study were performed on formalin-fixed paraffin embedded tissue sections using break-apart SYT probe, which hybridized to target the breakpoint gene. Troubleshooting for FISH were carried out in obtaining positive t(X;18) signal in SS cells.

Results: From IHC analysis, 74.1% (20 cases) of synovial sarcoma showed positive nuclear immunoreactivity to TLE-1. Strong nuclear immunoreactivity (3+) was 48.% and moderate nuclear immunoreactivity (2+) was 25.9%. Seven cases (25.9%) were negative to TLE-1. Weak nuclear immunoreactivity (1+) was 7.4% and no nuclear staining (0) was 18.5%. TLE-1 expression was not statistically significant with tumour morphology subtypes. Due to poor t(X;18) FISH signal, several troubleshooting plans were carried out i.e. pretreatment step, enzyme digestion and hybridization step, which the steps are known to be very sensitive to temperature, time and pH.

Conclusion : TLE-1 is a useful marker in diagnosing SS and to distinguish it from its histological mimickers. The final diagnosis of SS is only by pathologist eyes, as we still rely on morphology and IHC interpretation. The presence of agreement between TLE-1 IHC and its gold standard test (FISH) is a ticket for not to proceed with the later, which is more laborious and expensive. However, failure of signal detection due to technical and inaccurate methodology, we were unable to proof the agreement.

Keywords: *TLE-1, Immunohistochemistry, Fluorescence In Situ Hybridization, synovial sarcoma*

3.3 INTRODUCTION

Synovial sarcoma (SS) is a rare aggressive variant of soft tissue tumour, which comprises approximately 10% of the soft tissue sarcoma (Foo *et al.*, 2011).This mesenchymal neoplasm has gained an interest due to its delayed diagnosis, higher incidence of late metastases, poor baseline prognosis and lack of effective targeted therapy (Wisanuyotin *et al.*, 2013). To date, molecular analysis by FISH remains as the gold standard in diagnosing SS. Translocation t(X;18)(p11.2;q11.2) of SS18-SSX fusion gene has been remarked as a clinical diagnostic marker for SS (Chuang *et al.*, 2013) (Minami *et al.*, 2014) (Przybyl *et al.*, 2012) . However, procurement of expensive lab materials and high-end facilities limit the use of FISH analysis for tumour monitoring of SS.

To date, the final diagnosis of SS relies on morphology and IHC interpretation. Histology assessment classifies SS into three morphological subtypes; monophasic, biphasic and poorly differentiated (Colwell *et al.*, 2002) (Moberger *et al.*, 1968). These diverse morphology patterns often lead to diagnostic difficulty for SS. A panel of IHC markers i.e. Bcl-2, epithelial membrane antigen (EMA), cytokeratin, CD99, CD34, S-100 protein, and desmin, have been used to differentiate SS mimickers (Villaroel-Salinas *et al.*, 2012). However, the markers lack sensitivity and specificity to monophasic and poorly differentiated subtypes of SS (Chuang *et al.*, 2013). Transducer-like enhancer of split 1 (TLE-1) has been discovered as a robust marker with higher sensitivity and specificity to characterise SS subtypes (Knösel *et al.*, 2010) (Rekhi *et al.*, 2012). Lan *et al.* (2016) found that the sensitivity and specificity of TLE1 are 91.7 % and 94.9 %, respectively. The result suggest that TLE-1 may be a promising prognostic biomarker against SS malignancy. However, conflicting data exists on TLE-1 prognostic effect in SS due its variable positive expression.

Our major goal is to determine the validity of TLE-1 IHC marker by evaluating the agreement between this marker with FISH, which is considered as gold standard in diagnosing SS. We investigate the clinicopathological characteristic of SS patients, expression of TLE-1 in human SS phenotype using IHC and translocation X;18 using FISH analysis. However, we were unable to produce any positive signal for t(X;18) via FISH analysis in this study. This paper describes troubleshooting plans for FISH analysis that were carried out in determining positive signals for t(X;18).

3.4 MATERIAL AND METHODS

Synovial sarcoma specimens

This was a cross-sectional study carried out at Hospital Universiti Sains Malaysia from a period of 8 months from October 2016 to July 2017. Ethical clearance for the study was obtained from the Ethical Committee, Universiti Sains Malaysia (JEPEM Code : USM/JEPeM/16050189). A total of 27 cases diagnosed histologically as Synovial sarcoma (SS), were retrieved from registry book and computerized registry data files were selected. Representative paraffin block of the tumours were utilized in this study. In addition, the clinicopathological data for all selected cases were studied. The clinicopathological data i.e. age, clinical presentation, site and depth of tumour involvement, tumour size, presence of 5 years local recurrence and metastasis at diagnosis were retrieved from the original archieved formal pathology report and patients' record notes.

Histology assessment

The tissue slides were deparaffinized and hydrated using normal procedures and stained with hematoxylin and eosin (H&E). The slides were viewed using standard light

microscopy to determine the histological subtypes of the tumour i.e. monophasic, biphasic and poorly differentiated type.

Immunohistochemical (IHC) staining for TLE-1

IHC staining was performed to the paraffin-embedded tissue blocks according to standard procedures (Abcam Code Ab131648). The tissue slides were heated on hotplate at 60^{o}C for 1 hour. Sections were deparaffinized in xylene and rinsed in graded alcohols. Endogenous peroxidase was blocked by incubation in 3% hydrogen peroxidase for 5 minutes, followed by rinsing in distilled water. The sections were subsequently subjected to antigen retrieval by immersing in Tris-EDTA buffer solution (pH 9), followed by heating in a pressure cooker (DAKO, Denmark) for 3 minutes. Polyclonal primary antibody TLE-1 at 1:150 was applied to the sections and incubated for an hour at room temperature. The slides were washed in tris buffer saline (TBS) solution, pH 7.6 for 2 times. Subsequently, horseradish peroxidase (HRP) polymer (DAKO) was applied on each section and incubated for 30 minutes at room temperature. Slides were washed twice in TBS solution. Di-amino benzidine (DAB) from DAKO, a chromogen was applied to the sections and incubated for 5 minutes, followed by counterstaining with hematoxylin. Finally, the slides were mounted with DPX solution. Sections without the primary antibodies staining served as negative controls and these were run with each batch of staining together with positive controls for each antibody. Tissue from tonsil was used as positive control for TLE-1 staining.

Staining characteristic and scoring

TLE-1 expression was quantified in the various samples examined using a scoring method utilised previously by (Chuang, 2013). The percentage of nuclear staining was determined at a magnification of 400x, and scored 0/no staining (< 5% cell positive),