PRELIMINARY ANALYSIS OF STIM-1 EXPRESSION ON FORMALIN-FIXED PARAFFIN-EMBEDDED NASOPHARYNGEAL CANCER TISSUES

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Abstract

Nasopharyngeal cancer (NPC) is commonly diagnosed at the advanced stage with a poor prognosis. STIM-1 has become a promising cancer biomarker, especially in early diagnostic applications. There is a limited study on STIM1 expression in NPC, especially in formalin-fixed paraffin-embedded NPC tissues. Thus, the present work provides a preliminary analysis of STIM-1 expression on NPC tissues. This study aims to examine STIM-1 expression in NPC associated with tissue origin and the functional effects of STIM1 expression in NPC. Screening for target genes was using the KEGG PATHWAY database. The target gene analysis was initially done further studied using an immunohistostaining approach on NPC tissue samples. From the bioinformatic resources, STIM-1 has shown significant interaction with molecules network associated with cell migration and metastasis. Differentiated NPC showed moderate STIM-1 IHC staining intensity and undifferentiated NPC presented with strong STIM-1 IHC staining intensity. The present preliminary study suggests that STIM-1 could have a positive correlation with NPC pathogenesis. However, further comprehensive work using larger samples size is needed especially focusing on the STIM-1 expression and other clinicopathological parameters.

Keywords: Nasopharyngeal cancer, Nasopharyngeal carcinoma, Formalin-fixed paraffin-embedded, STIM-1, Cancer biomarker, Diagnostic, Immunohistostaining

Introduction

According to differentiation level and microscopic histological appearance, NPC is divided into three subgroups (Table 1) (1). The WHO's classifications are as follows: The creation of keratin protein distinguishes keratinising squamous cell carcinoma, also known as type 1, differentiated non-keratinising carcinoma, generally known as type 2, and undifferentiated carcinoma, popularly known as type 3 (2-4). Type 1 NPC exhibits keratinisation characteristics with intercellular bridges and squamous histologically (5). This sort of NPC is described as poorly or moderately differentiated. Types 2 and 3 are more prevalent and strongly associated to the Epstein-Barr virus (EBV),

especially in Asia, while type 1 is uncommon in areas where NPC is endemic (5, 6). Similar to undifferentiated NPCs in terms of histological appearance, non-keratinising differentiated NPCs have former cell borders that are stratified and pavemented (3). Undifferentiated NPC has vesicular hyperchromatic nuclei that are in the shape of spindles or ovals and have protruding nucleoli and mitotic activity (8). However, the stage of the malignancy determines the survival rate of NPC patients (Table 1). For instance, patients with stage I or stage II NPC, have a 5-year survival rate of 72%-90%, whereas for stage III, it is only 55%. Due to frequent locoregional metastasis or recurrence, stage IV NPC patients have a 30% survival rate (9, 10).

Table 1: NPC classification and stages

Classification of NPC (Barnes et al., 2005)		
WHO Classification 1978	WHO Classification 1991	WHO Classification 2005
WHO type I (well-differentiated keratinised SCC)	Squamous cell carcinoma	Keratinising squamous cell carcinoma (SCC)
WHO type II (differentiated keratinised non-SCC)	Non-keratinising carcinoma (a) Differentiated (b) Undifferentiated	Non-keratinising carcinoma (a) Differentiated (b) Undifferentiated
WHO type III (undifferentiated carcinoma)		Basaloid squamous cell carcinoma

Stages of NPC and treatment (Lee et al., 2005)		
Stage I	Stage II, III, IVA and IVB	Stage IVC (distant metastasis)
Definitive treatment Radiotherapy to nasopharynx & Radiotherapy to neck	Concurrent chemotherapy and radiotherapy	Palliative chemotherapy Radiotherapy to alleviate symptoms
IMRT to minimise dose to critical structure	IMRT to minimise dose to critical structure	Referral to palliative care/ palliative home care

NPC at an advanced stage presents with challenging prognosis and treatment options (11, 12). By focusing on STIM-1, researchers might learn more about the pathophysiology of NPC, its molecular impacts, particularly oxidative stress, and possible molecular targets that might prevent or lower the incidence of NPC. NPC has a poor prognosis because of late lesions, which arise from a lack of knowledge on the molecular etiology. The oxidative stress responses are associated with the etiology of malignancies and ineffective treatments (13). According to a few previous studies, STIM-1 dysregulation has been associated to cause hyperactivities in malignancies, including cell proliferation, cell cycle disruption, migration, and apoptosis inhibition, according to earlier investigations (14,15). Concomitant chemotherapy and radiation therapy are the recently available treatment options for NPC. Nevertheless, treating NPC at a late stage is difficult and has a high mortality rate. With 2.222 new cases, 1.450 deaths, an age-standardized rate of 6.3 per 100,000, and an age-standardized mortality rate of 3.7 per 100,000, NPC was the fourth most prevalent cancer in Malaysia in 2020. (16). To date, the STIM-1 expression in NPC tissues in locally reported cases is not clear. Present work performed preliminary analysis on STIM-1 expression on NPC Formalin-Fixed Paraffin-Embedded (FFPE) tissue specimens based on locally reported cases.

Experimental Design

Screening for target genes via KEGG pathway

Kyoto Encyclopedia of Genes and Genomes (KEGG) project was initiated in 1995 by Japanese researchers, as part of Human Genome database (17). The Human-Genome project was initiated to reveal the human genetic factors associated with various diseases and to develop new preventive, diagnostic, and treatment strategies (18). It is a database for genomic knowledge which helps in the systematic assessment of gene functions and connects functional information with genomic information. The GENE database stores genomic information via the collection of gene catalogs for all sequenced genomes with up-to-date functional annotation. The KEGG pathway database stores higher functional information showing graphical descriptions of the cellular processes, including, signal transduction, membrane transport, cell cycle and metabolism (19).

KEGG uses Java-graphics tools to browse and compare genome maps, manipulate of expression maps, and serve as a computational tool for comparing sequences and graphs as well as path computation. In order to comprehend the intricate picture of the disease in terms of correlations between gene networks and the disease, the KEGG network (http://www.genome.ad.jp/kegg/) was examined. The network depicts the network components used to identify human genes (IDs). The variant genes networks of the associated molecular networks were selected using the components of the variant network. To identify the various genes involved in various biological pathways, KEGG pathway analysis (https://www.genome.jp/kegg/pathway) was performed. The KEGG database resource connects molecular data from genome sets to the networks (pathways) of molecules that describe systemic activities.

Immunohistochemistry (IHC) staining

The ethical clearance was approved by Human Research Ethics Committee of Universiti Sains Malaysia (USM/JEPeM/20030182). Three formalin fixed-paraffin embedded (FFPE) NPC tissue samples and Colorectal cancer (CRC) tissue were collected from the pathology

department of Advanced Medical and Dental Institute, Universiti Sains Malaysia, and a microtome was used to section them. Colorectal cancer (CRC) tissues present positive immunohistostaining for STIM-1 overexpression (20). The slides were heated in an oven for 10 minutes at 60°C before being immersed in Xylene (Sigma) for 5 minutes to deparaffinized the NPC FFPE tissue sections. The next step is to hydrate which they were passed through absolute ethanol twice for five minutes each, followed by passing it through 95% ethanol twice for five minutes each, before dipping through 70% ethanol for five minutes, and next into the diH2O for five minutes. The slides were added Envision Flex target retrieval solution which was then heated in a microwave for 20 minutes (pH 6.0) while the concentration of the retrieval solution was continuously monitored in order to retrieve antigen. After being chilled at room temperature for 20 minutes, the slides were washed for 5 minutes and thrice each using Envision Flex wash buffer. The slices were subjected to a peroxidase-blocking solution for 5 minutes and incubated at 4°C overnight with STIM-1 primary antibody (1:100).

Envision Flex wash buffer (Dako) was used to wash the sections three times for five minutes each. Then, they were covered with Envision Flex HRP and left for 20 minutes at room temperature. The wash buffer was used to wash the tissue sections. They were wash for five minutes thrice each after being incubated with Envision flex substrate working solution for one minute (1 drop of DAB+ chromogen to one ml of substrate buffer). The sections were counter-stained by incubating with Envision Flex Haematoxylin for 5 minutes, washing with wash buffer and distilled water for 5 minutes each, and then rinsing twice with tap water. After being air-dried, the slides were mounted using the mounting media. Before mounting on the mounting medium, the slides were air-dried. The phase contrast microscope (Olympus CKS40) was used to generate microscopic pictures (20x objective lens, toolbar 50nm) of the immunohistochemically stained tissues, which revealed STIM-1 proteins that were stained brown.

Results
Biomarker analysis

The understanding of STIM1 molecular function and biological pathways was assessed by the KEGG bioinformatics platform. STIM-1 interacts with ORAI-1 channels and generates SOCE, which encourages the activation of several Ca2+-dependent signaling molecules and pathways and ultimately causes cell death, migration, or proliferation (Figure 1).

STIM-1 expression study on FFPE tissues

STIM-1 protein expression was observed in non-keratinising differentiated and two undifferentiated NPC tissues (Figure 2). An immunohistochemistry-positive control of STIM-1 overexpression was presented by colon cancer tissue (Figure 2A) as reported previously (20). Strong expression of the brown staining marker, STIM1 protein was observed. Non-keratinising differentiated NPC (Figure 2B) exhibited moderate staining intensity and non-keratinising undifferentiated NPC presented with strong staining intensity (Figures 2C and D) as the positive control (Figure 2A).
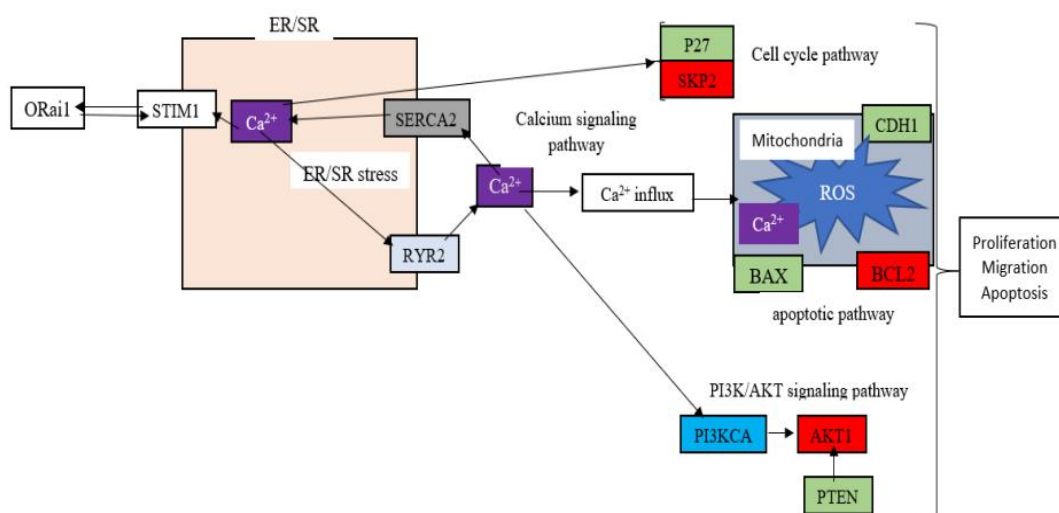

Figure 1: STIM-1 and its associated genes/pathways from KEGG. Cell migration, proliferation, and apoptosis are induced by the activation of several Ca2+-dependent signalling molecules and pathways by STIM-1 proteins that activate ORAI-1 channels.
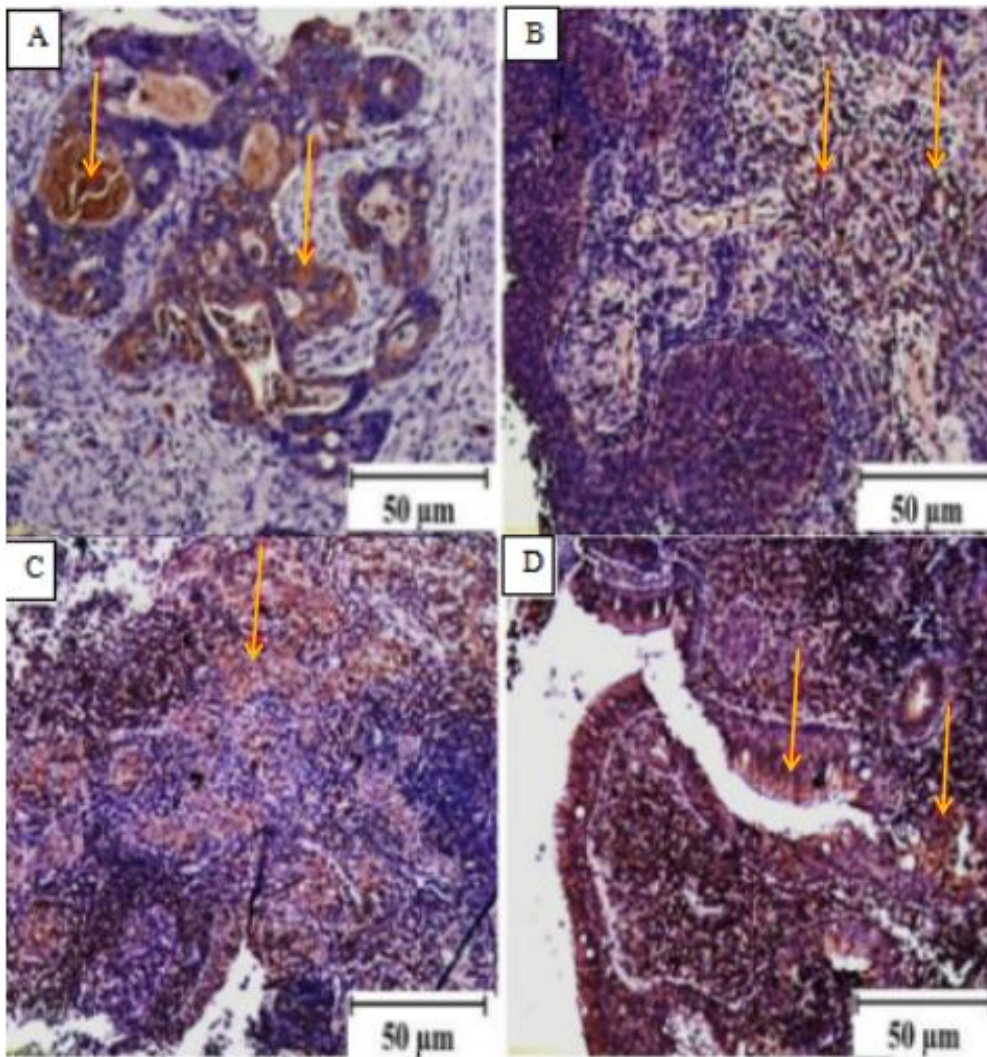
Figure 2: STIM-1 expression on NPC tissues. The immunohistochemically stained tissues showed brownish immunohistochemical stained STIM-1 proteins under (20x objective toolbar 50nm). (A) Strong STIM-1 IHC staining intensity in colon cancer that serve as positive control for STIM-1 over expression. (B) Moderate STIM-1 IHC staining intensity in non-keratinising differentiated NPC. (C and D) Strong STIM-1 IHC staining intensity in non-keratinising undifferentiated NPC. Magnification, 20×. Scale bars are 50μm.

Discussion

KEGG pathway revealed ORAI-1, STIM-1, RYR2, and SERCA are all part of the calcium (Ca2+) signaling pathway (Figure 1). Ca2+ fluxes are produced by the entry of Ca2+ from the outside into the cell through voltage-operated channels (VOCs) across the membrane, which controls cellular functions. A significant additional source of Ca2+ is the ER/own SR's Ca2+ reserves. The major Ca2+ source is from the internal Ca2+ stores and from those in the ER/SR. Inositol-1,4,5-trisphosphate- or ryanodine-receptors control the release of Ca2+ from the ER/SR (RYRs). STIM-1 detects Ca2+ depletion in the ER/SR microenvironment while Ca2+ is the primary channel activator.

In general, oxidative stress and calcium homeostasis are regulated by the calcium pathway. Cell invasion and apoptosis also involves in the calcium signaling system. AKT, PIK3CA, PTEN, and other genes that control cell growth and apoptosis are part of the PI3k/Akt signaling pathway. The results of the KEGG analysis showed that STIM-1 is associated to genes involved in the cell cycle, including SKP2 and P27. Additionally, STIM-1/Ca2+ controls BAX and BCL2 through the regulation of oxidative stress in the mitochondria, which cause cell death and controls cell adherence via CDH1.

The present research, which focused on local (Malaysian) NPC tissue cases, discovered a positive correlation between STIM1 and NPC pathogenesis. The non-keratinising differentiated NPC demonstrated a medium level of STIM1 staining intensity (Figure 2B). Non-keratinising undifferentiated NPC (Figures 2C and 2D) demonstrated strong STIM1 staining. According to previous research, STIM-1 overexpression in CRC tissues correlates with tumour growth, invasion, and metastasis (21). Based on the results of this study, STIM-1 overexpression in NPC might be associated with

high potent metastatic behaviour (20). The finding suggests that STIM-1 expression is high in NPC tissues and varies according to the tumour type.

Conclusion

Present preliminary work has found that STIM-1 expression higher in non-keratinising undifferentiated NPC could be associated with tissue subtype and its pathogenesis profile. A further comprehensive evaluation of STIM-1 association between different NPC subtypes is needed especially using larger samples size. Knowledge from these studies could strengthen the understanding of STIM-1 impact on NPC tumorigenesis and its therapeutic potential.

Acknowledgements

Competing interests

The authors declare no competing interests.

Availability of data

All data for this study are available via the corresponding author upon reasonable request.

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