Sep 25, 2024

The "IHC and TMA staining, PDO cultivation and dose-resopnse assay" section of the manuscript entitled "The prognostic value of H3K27me3 implies potential therapeutic targets of EZH2 in sinonasal soft tissue sarcoma: evidence gained from an single-arm, prospective, observational trial interim analysis"

This protocol is a draft, published without a DOI.

Chengle Zhou[1], Jingyi Yang[2]

[1]Eye & ENT Hospital of Fudan University; [2]Eye & ENT Hospital, Fudan University, China

ENT-Hongmeng Yu

Chengle Zhou
Eye & ENT Hospital of Fudan University

OPEN ACCESS

Protocol Citation: Chengle Zhou, Jingyi Yang 2024. The "IHC and TMA staining, PDO cultivation and dose-resopnse assay" section of the manuscript entitled "The prognostic value of H3K27me3 implies potential therapeutic targets of EZH2 in sinonasal soft tissue sarcoma: evidence gained from an sin. **protocols.io https://protocols.io/view/the-34-ihc-and-tma-staining-pdo-cultivation-and-do-diwq4fdw**

Manuscript citation:
The manuscript concerned was currently in the process of submission.

Protocol status: Working
We use this protocol and it's working

Created: August 12, 2024

Last Modified: September 25, 2024

Protocol Integer ID: 105136

Keywords: endoscopic surgery, epigenetics, H3K27me3, sinonasal malignancies, sinonasal soft tissue sarcoma

Funders Acknowledgement:
National Natural Science Foundation of China
Grant ID: 81970856, 82371123
National Key Clinical Specialty Construction Project
Grant ID: Z155080000004
Shanghai Science and Technology Committee Foundation
Grant ID: 21JC1401100, 23Y31900500
the New Technologies of Endoscopic Surgery in Skull Base Tumor: CAMS Innovation Fund for Medical Sciences (CIFMS)
Grant ID: 2019-I2M-5-003
Shanghai Municipal Key Clinical Specialty
Grant ID: shslczdzk00801
Outstanding Doctor & Outstanding Clinical Researcher" project supported by the Eye, Ear, Nose and Throat Hospital of Fudan University
Grant ID: SYB202006
Shanghai Shen Kang Hospital Development Center
Grant ID: SHDC22021214
Science and Technology Commission of Shanghai Municipality
Grant ID: 20Y11902000
Science and Technology Commission of Shanghai Municipality
Grant ID: 21ZR1411700
National Natural Science Foundation of China
Grant ID: 82303862

Disclaimer

No disclaimer

Abstract

The manuscript entitled "**The prognostic value of H3K27me3 implies potential therapeutic targets in sinonasal soft tissue sarcoma: evidence gained from a single-arm, prospective, observational trial interim analysis "** originated from exploration of a single-am, prospective, observational trial conducted in sinonasal STS patients evaluating the clinical efficacy of endoscopic surgery, and firstly aimed at identifying for the first time the prognostic value of an epigenetic biomarker H3K27me3 in sinonasal STS patients . Secondly, a clinical applicable nomogram model for risk stratification was constructed and validated. Thirdly, in pursuit of clinical translation, since cell lineages, drug testing platforms and innovative therapies is scarce in sinonasal STS, we employed patient derived organoids(PDO) as preclinical models, and probed novel therapeutic innovations through PDO-based drug-response assay.
Meanwhile, the current protocol webpage at protocols.io encompassed the following methodological sections of the exact manuscript: the IHC and TMA staining, PDO cultivation and dose-resopnse assay. This webpage was constructed for acdemic discussion and methodoligical deposit.

Guidelines

The current protocol webpage at protocols.io encompassed the following methodological sections of the exact manuscript: the IHC and TMA staining, PDO cultivation and dose-resopnse assay. This webpage was constructed for acdemic discussion and methodoligical deposit.

Materials

1. Materials concerned in the process of "Construction of Tissue microarrays"

a. Whole slides staining of 27 formalin-fixed, paraffin-embedded (FFPE) tumor sample

b. Tissue microarrays (TMA) where FFPE samples were blocked and cut into sections of 4 μm thickness

2. Materials concerned in the process of "Immunohistochemistry staining and evaluation"

a. primary antibodies were employed for immunostaining: H3K27me3 (MXB biotechnologies, RM175, 1:100), Ki-67 (abcam, ab15580, 1:4000), EGFR (Abcam, ab52894, 1:100), PD-L1 (Abcam, ab237726, 1:100), CD34 (Abcam, ab81289, 1:2000), BRG1 (Gene Tech, GR005, 1:100), EZH2 (CST, 5246, 1:100), INI1 (Origene, ZA-0696, 1:100).

3. Materials concerned in the process of "Immunofluorescent staining"

a. 5 mm FFPE slide of tumor specimen

b. Reagents used in the chronological order during the tyramide signal amplification assay: 10% formalin, Antigen Retrieval Buffer, 5% BSA (Sigma, B2064)(for blocking), primary antibodies for EZH2 staining: EZH2 (CST, 5246, 1:100), secondary antibodies for EZH2 staining: goat anti-rabbit IgG H&L (HRP) antibodies (Abcam, ab205718, 1:2000), tyramide reagent Try-488(Runnerbio Biotech. Comp. (Shanghai, China)), primary antibody for H3K27me3 (MXB biotechnologies, RM175, 1:100), tyramide reagent Tyr-cy5(Runnerbio Biotech. Comp. (Shanghai, China)). The whole staining process can be briefly summarized as: EZH2-Try-488, and H3K27me3-Try-cy5. Subsequently, Slides were stained using anti-fade mounting medium with DAPI (Beyotime Biotechnology, Shanghai, China)

4. Materials concerned in the process of "Operation process of PDO model construction"

a. Reagents used in PDO cultivation: tissue cleaning solution (Beijing Daxiang Technology, KS100126), tissue enzymatic digestion solution (Beijing Daxiang Technology, KS100128, KS100130), organoid cleaning solution (Beijing Daxiang Technology, KC100141), matrix gel (Beijing Daxiang Technology, MG100101), 24-well cell culture plates, organoid culture medium (Beijing Daxiang Technology, OK100224), anti-apoptotic factors (Beijing Daxiang Technology, IA100101), organoid cleaning solution(Beijing Daxiang Technology, RKC100141)

5. Materials concerned in the process of "PDO drug response assay"

a. Reagents used in PDO drug response assay: 60% concentration matrix gel, micro-wells of the IBAC S1 chip, cell culture medium containing different drug schemes and diluted concentrations through a three-fold gradient, DMSO (positive control) and staurosporin (5 μM Staurosporine, MCE) (negative control), CellTiter-Glo 2.0 assay (Promega, G9243) , GraphPad Prism (version 8.0).

Safety warnings

No warnings.

Ethics statement

The one-arm, observational, prospective clinical trial with the aim to explore the postoperative treatment outcomes of endoscopic surgery in sinonasal STS patient was registered in the Chinese Clinical Trial Registry with the identifier of ChiCTR2400088405 (URL:https://www.chictr.org.cn/bin/project/edit?pid=236269).
The ethnical approval of the prospective trial was conducted by the Ethics Committee of Eye & ENT Hospital of Fudan University (2021099). Written informed consent was signed by all patients.

1. Construction of Tissue microarrays

1 Whole slides staining of 27 formalin-fixed, paraffin-embedded (FFPE) tumor samples was firstly conducted utilizing available surgical specimen obtained from the observational trial cohort for primitive discovery. For subsequent validation, remaining FFPE specimens of the previous 27 individuals coupled with FFPE of 18 retrospectively recruited samples were gathered for tissue microarray (TMA) construction. Prior to TMA construction, patients' hematoxylin and eosin stained slides were reviewed by an experienced pathologist to confirm the existence of tumoral areas. The cores of representative tumor area of each patient were drilled out of the original FFPE blocks, with a maximum diameter of 1.5 mm, based on the size of primary tumors. Tumor blocks were then embedded into TMA blocks and cut into sections of 4 μm thickness, which were serially mounted on glass slides.

2. Immunohistochemistry staining and evaluation

2 The following primary antibodies were employed for immunostaining: H3K27me3 (MXB biotechnologies, RM175, 1:100), Ki-67 (abcam, ab15580, 1:4000), EGFR (Abcam, ab52894, 1:100), PD-L1 (Abcam, ab237726, 1:100), CD34 (Abcam, ab81289, 1:2000), BRG1 (Gene Tech, GR005, 1:100), EZH2 (CST, 5246, 1:100), INI1 (Origene, ZA-0696, 1:100).
During semi-quantification of H3K27me3 staining, H3K27me3 was observed to have an incomplete loss status, consistent with previous meningeoma findings[24–26]. Four gradational expression patterns were revealed, where H3K27me3 loss was identified in 0-25%, 25-50%, 50-75%, and 75%-100% of tumor cells, respectively. In our study, H3K27me3 was assigned as "impaired status" if loss of H3K27me3 appeared in greater than 50% of tumor cells, while H3K27me3 positive was addressed with complete positive staining or less than 50% staining loss[27].
Evaluation of PD-L1 expression was assessed by combined positive score (CPS) [28], defined as the ratio of PD-L1-stained cells (tumor cells, lymphocytes and macrophages) out of total tumor cells multiplied by 100, with CPS>=1 considered positive. The expression of EGFR was semi-quantitatively determined according to the following criteria[29]: 0, no or faint staining intensity in < 10% of tumor cells; 1+, faint cytoplasmic staining in 10% of tumor cells; 2+, moderate and incomplete membranous staining in 10% of tumor cells; and 3+, strong membranous staining in 10% of tumor cells. For angiogenesis quantification. Microvessel density (MVD) was assessed by immunostaining with the anti-CD34 antibody. In brief, 4 vascular hot spots were identified by scanning each TMA cylinder at low magnification (x100), and then the MVD of each specimen was defined as the average vessel count per visual field of 4 vascular hot spots at high magnification (x200), which was then classified into 2 groups: "MVD high" and "MVD low", with the median MVD value (8.63) as the cutoff[30].
EZH2 Expression[31] was assessed using the proportion of stained cells, and categorized into strong expression (≥50% expression) and moderate expression (≥25% cells) and weak expression (<25% cells). BRG1 and INI-1 expression was assessed via immunostaining of the tumor nuclei, and loss of nuclear staining with positive staining of endothelial cells as internal control were considered BRG1 and INI-1 deficient, respectively[32, 33]. The Ki-67 index was

calculated by the percentage of positively stained tumor cells among all tumor cells[34], with an optimal cut-off value of 27.5%, obtained through receiver operating characteristic curve (ROC) analysis, segregating the study cohort into Ki67-high (≥27.5%) and Ki67-low (<27.5%) groups.

3. Immunofluorescent staining

3 Tyramide signal amplification assay was employed for multiplexed Immunofluorescence of the multi-refractory sinonasal STS individual opted for PDO development. Localization of EZH2 and H3K27me3 was examined using FFPE tumor specimen.
5 mm FFPE slide was put through two sequential rounds of staining. After heated at 65 degrees for 1hr, all slides were deparaffinized and tissues were fixed with 10% formalin prior to antigen retrieval in heated Antigen Retrieval Buffer for 18 min in Retriever microwave. Tissues were then blocked with a 5% BSA (Sigma, B2064) for 20 min before an 1h incubation at room temperature with respective primary antibodies: EZH2 (CST, 5246, 1:100) . After 3 times PBS wash, 30 min incubation of secondary antibodies of goat anti-rabbit IgG H&L (HRP) antibodies (Abcam, ab205718, 1:2000) were conducted at room temperature, and underwent 3 times PBS wash. After adding **tyramide reagent** Try-488, the second primary antibody H3K27me3 (MXB biotechnologies, RM175, 1:100) was added and the staining process was replicated as the second round until the addition of another **tyramide reagent** Tyr-cy5. Try-488, and Try-cy5 reagents were purchased from Runnerbio Biotech. Comp. (Shanghai, China). The whole staining process can be briefly summarized as: EZH2-Try-488, and H3K27me3-Try-cy5. Samples were covered with anti-fade mounting medium with **DAPI** (Beyotime Biotechnology, Shanghai, China) and captured using the pannoramic digital slide scanners (PANNORAMIC Scan II, 3DHISTECH, Hungary).

4. Operation process of PDO model construction

4 Tumour tissues from the case study individual (case number: STS-096) were cut into 1-2 mm^3 piecemeal blocks, washed with tissue cleaning solution (Beijing Daxiang Technology, KS100126), and digested using tissue enzymatic digestion solution (Beijing Daxiang Technology, KS100128, KS100130) for 30-60 minutes at 37°C. Cells were then resuspend using organoid cleaning solution (Beijing Daxiang Technology, KC100141), passed through a 100μm filter, and then centrifuged (800g for 5 minutes). After centrifugation, cells were resuspended in matrix gel (Beijing Daxiang Technology, MG100101), and seeded in droplets onto 24-well cell culture plates. After gel solidification in a cell culture incubator, 500μl of organoid culture medium (Beijing Daxiang Technology, OK100224) was added. During initial 2 days, the organoid culture medium was supplemented with anti-apoptotic factors (Beijing Daxiang Technology, IA100101). Cell medium is refreshed every 3-4 days, and passaging is carried out every 7-14 days based on the growth status of tumoroids in the following manner: organoids were harvested, centrifuged, digested using organoid cleaning solution(Beijing Daxiang Technology, RKC100141) followed by mechanical dissociation of the organoid mixture. The dissociated organoids were washed again with organoid cleaning solution, then centrifugated and resuspended using matrix gel as the primary cultivation method described above.

5. PDO drug response assay

5 In IC50 evaluation of each drug scheme, the digested organoids are collected and thoroughly mixed with 60% concentration matrix gel, and then seeded into micro-wells of the IBAC S1 chip with the concentration of 1000-2000 cells/5 μL/well. Briefly, PDOs were incubated with cell culture medium containing different drug schemes and diluted concentrations through a three-fold gradient, with DMSO and staurosporin (5 μM Staurosporine, MCE) served as positive and negative control, respectively. After 96 hours of drug incubation, cell viability was quantified using the CellTiter-Glo 2.0 assay (Promega, G9243) for measurement of luminescent ATP signal. Results were then normalized to the negative control and displayed in the percentage of cell viability form. Dose-response curves were plotted using GraphPad Prism (version 8.0).

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