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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 "Material and Methods" methodological sections of the exact manuscript. This webpage was constructed for acdemic discussion and methodoligical deposit.

Guidelines

The current protocol webpage at protocols.io encompassed "Material and Methods" methodological sections of 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"**. 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.

Before start

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"** was currently under review. For inquiry, please contact the scripting author Chengle Zhou, M.D. via email: chenglezhou@126.com.

1. Overall Study Design

1 This study brought an interim report of a one-arm, observational, prospective study to explore the postoperative treatment outcomes of endoscopic surgery in sinonasal STS patient (URL:https://www.chictr.org.cn/bin/project/edit?pid=236269; Identifier: ChiCTR2400088405). The primary endpoint of the study is the OS. The secondary endpoints include the LRFS and HRQoL. Exploratory outcomes included investigating and assessing prognostic indicators for clinical outcome prediction in sinonasal STS patients, as well as to establish predicting models for clinical outcome evaluation. The design of this prospective study is available on chictr.org. The study was retrospectively registered on 19 August 2024. The study is still ongoing, with 40 individuals as an estimated sample size and 5-year as a rated follow-up duration. We are presenting an interim report to show phase results of the prospective trial by revealing treatment outcomes of sinonasal STS after endoscopic resection, with exploration to search for prognostic indicators and potential drug targets.
Within the framework of prospective data collected from this trial, retrospective analysis of prognostic biomarkers was carried out using a tissue microarray (TMA) cohort with larger sample size and expanded length of follow-up duration, to validate the findings on survival predictive biomarkers. Multivariate regression models were constructed for survival analysis, which resulted in the establishment of a nomogram integrating the prognostic biomarker and the routinely used AJCC system. At last, we explore a process for individualized therapeutic regimen screening for sinonasal STS, which represents as a rare, heterogeneous, and sometimes refractory disease, by synergistic application of patient-derived organoids (PDO) based pre-clinical models.

2. Participants

2 Eligible patients receiving endoscopic surgery were screened and recruited to the prospective cohort from 10 August 2021 to 10 October 2023 at the Eye & ENT Hospital of Fudan University, Shanghai, China. The inclusion criteria were as follows: (1) histologically diagnosis of sinonasal STS confirmed by two independent pathologists; (2) no distant metastasis; (3) presence of malignancy considered possible of complete resection via endoscopic approach by ENT surgeons; (4) relatively good physical condition with tolerance of endoscopic surgery; (5) proper functioning of the major organs: Eastern Cooperative Oncology Group PS of 0–1; (6) informed consent to participate in the study. Exclusion criteria were as follows: (1) distant metastasis; (2) presence of contraindications for surgery, including cardiopulmonary diseases, coagulation dysfunction; (3) with uncontrolled infection or other concurrent diseases; (4) with concurrent acute or chronic psychiatric disorders; (5) pregnancy or lactation; (6) with any other circumstances that may hinder research compliance. This prospective study was approved by the Ethics Committee of Eye & ENT Hospital of Fudan University (2021099). Written informed consent was signed by all patients.

The patients' FFPE specimen were retrospectively retrieved for TMA cohort construction from our skull-base tumor sample collection. 18 sinonasal STS patients whose FFPE samples were collected during endoscopic surgery from 10 August 2021 to 18 December 2023, with confirmed histological diagnosis of sinonasal STS. All participants signed written informed consent to provide tumor specimen.

3. Treatment procedures

3 Baseline characteristics of the prospective cohort were collected from the case report forms, while patient information of retrospective cohort was retrieved from documented medical record. We collected the sex information of participants determined by biological sex (female or male). The invasion status, staging of tumor and lymph node metastases were determined according to MRI and contrast-enhanced CT images. Metastatic lymph nodes were identified using the following criterion: diameter>1.5cm, rounded shape, disappeared lymphatic portal, and central necrosis. Orbital invasion was categorized via the Iannetti grading [21]: (I) erosion or destruction of the medial orbital wall; (II) invasion of the periorbital fat tissue; and (III) invasion of the medial rectus of the ocular bulb, the ocular bulb itself, the optic nerve, or the palpebral skin. Treatment outcome of chemotherapy was evaluated based on the Response Evaluation Criteria in Solid Tumors version 1.1 (RECIST v1.1), with complete response (CR) defined as the disappearance of all lesions and pathological lymph nodes with a reduction in short axis to <10 mm, partial response (CR) characterized by at least a 30% decrease in the size of target lesions, and stable disease (SD) regarded as less than 30% remission in the size of target lesions. All histological subtypes were eligible according to postoperative pathological diagnosis reports.
A meticulous follow-up strategy was compiled for patients in the trial, with survival visits performed every 3 months for 2 years postoperatively, after which biannually for subsequent 3 years, until the observation of the event of interest (EOI): death due to any cause, withdrawal from the study, progression of disease, whichever occurred first. Contrast-enhanced CT or MRI of the affected area, nasal endoscopy and assignment of quality of life scale were obtained at each visit. OS was defined as the duration between the date of radical surgery and death from any cause. LRFS, DMFS and DFS refers to as the duration between the date of radical surgery and local recurrence, distant metastasis, and disease relapse at any site, respectively, or death, whichever occurred first. The last date for patient follow-up cut-off for this interim report was 18 December 2023.

4. Data collection, Data Evaluation and Follow-up Procedures

4 Baseline characteristics of the prospective cohort were collected from the case report forms, while patient information of retrospective cohort was retrieved from documented medical record. We collected the sex information of participants determined by biological sex (female or male). The invasion status, staging of tumor and lymph node metastases were determined according to MRI and contrast-enhanced CT images. Metastatic lymph nodes were identified

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5. Assessment of quality of life (QOL)

5 We employed EORTC-QLQ-C30 and EORTC-QLQ-H&N35, two well-validated instrument for HRQol assessment of core quality and head & neck cancer patients[22, 23] All PRO domains of two instruments have been elaborated in our study, and raw scores were rescaled within the range of 1 to 100, with higher values indicating better outcomes on GHS and physical functioning scale appeared only in EORTC-QLQ-C30 instrument, and poorer outcomes on symptom scales appeared in two instruments. Data were routinely collected at baseline, after surgery, and at each follow-up visit until the end of surveillance, and the analysis timepoint were determined at week 4 and week 12 postoperatively, with at least 50% of overall completion rate.

6. Construction and validation of the prognostic nomogram

6 A predictive nomogram for optimization of the conventional AJCC staging system incorporated AJCC T and N stage with the remaining variable of the stepwise regression model, H3K27me3 in the current study. The discriminative ability of the nomogram in 6-months and 1-year OS was assessed and compared with the conventional staging system via concordance-index with bootstrapped 1000 resamples and the time-dependent AUC of the ROC curves, respectively.

Real-world applications of our model lied in the risk stratification of postoperative sinonasal STS patients based on trisection of the summarized scores derived from the nomogram output.

7. Construction of Tissue microarrays

7 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.

8. Immunohistochemistry staining and evaluation

8 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.

9. Immunofluorescent staining

9 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).

10. Operation process of PDO model construction

10 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.

11. PDO drug response assay

11 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).

12. Statistical analysis

12 In report of the observational trial and the TMA results, categorical binary variables were compared using Fischer's exact test or the kai-square test. Survival curves were plotted in Kaplan-Meier curves and compared using the log-rank method. Hazard ratios and corresponding 95% CIs of each variable were summarized by Cox proportional hazard regression model. A two-tailed p value < 0.05 was considered significant.
To optimize the conventional staging system, a nomogram was constructed as a graphical projection model for computing survival probability. Receiver operator characteristic (ROC) curves, concordance index were employed to evaluate the predictive ability and performance of the
For PRO evaluation, the least-squares (LS) mean change from baseline from each PRO endpoint (week 4 and week12) was analyzed using the linear mixed-effects model for repeated measures, with change from baseline as the response variable, and baseline score, baseline score by study visit included as fixed effects. Separate models were constructed for each PRO variable, and least-squares mean changes from baseline to week 4 and week 12 were estimated with 95% CI.
The statistical analysis was conducted using R software (version 3.5.3; http://www.Rproject.org).

13. Role of funding source

13 The funders of the study had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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