S100A8/A9 Acts as Prognostic Indicators and Promotes Migration and Invasion via p38 MAPK Pathway in Nasopharyngeal Carcinoma

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Research

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Abstract

**Background** Nasopharyngeal carcinoma (NPC) is one type of malignancy associated with migration and invasion through a currently unclear mechanism. We previously discovered S100A8/A9 levels were roughly elevated in the plasma of NPC patients as the promising biomarkers. However, their expressions and underlying functions in NPC tissues are still unknown.

**Methods** In the present study, we analyzed 49 NPC tissues and 20 chronic pharyngitis (CP) tissues. Immunohistochemical staining was performed in different tissues and analyzed by mann-whitney U test statistically. Transwell migration and invasion experiments were further performed to determine S100A8/A9 effects on NPC.

**Results** Our results showed that S100A8/A9 in NPC tissues were significantly higher than those in CP tissues, closely associated with NPC clinical stages. Intriguingly, exogenous S100A8/A9 protein stimulation could dramatically enhance NPC migration and invasion abilities. In addition, p38 MAPK pathway blockade could diminish the migration and invasion of NPC cells stimulated by S100A8/A9 proteins. The downstream tumor invasion and migration associated proteins (e.g. MMP7) were also elevated in NPC tissues, consistent with S100A8/A9 overexpression.

**Conclusions** Taken together, our present findings suggest that the secreted soluble inflammatory factors S100A8/A9 might promote cancer migration and invasion via p38 MAPK signaling pathway along with invasion/migration associated proteins overexpression in the tumor microenvironment of NPC. This may shed light on the mechanism understanding and provide more novel clues for NPC diagnosis and therapy.

1. Introduction

Nasopharyngeal carcinoma (NPC) is the most common otorhinolaryngological tumor type originated from the upper skin. [1, 2] In 2018, the new incidence was estimated as 130,000 cases from the latest global cancer surveillance report. [3] NPC is well known as a cancer type characterized by distinct ethnic and regional specificity. [4] Endemic to China, this malignancy represents a variable occurrence rate ranging from the high incidence (Southern China) to a low rate (Northern China), while it is a rare disease among Caucasians. [4] Southern China (e.g. Guangdong, Guangxi) suffers a very high-risk of NPC, where the incidence ratio is much higher than the world’s. [5, 6] Non-keratinized low differentiation NPC with high degree of malignancy is the main pathological type in clinical. [7] As high as 60–70% of the newly diagnosed NPC patients have already developed to local advanced lesions. [8, 9] Along with the development of modern radiotherapy and chemotherapy, the initial treatment response rate currently reaches 90.9% for these NPC patients with advanced stages (III and IV stage), [10] and the five-year survival rate is 72.3–86%. [11–13] However, NPC often has a high incidence of recurrence and distant metastasis, especially for those patients with advanced stages. As for them, the five-year incidence is 20–30% [14, 15] and the ten-year incidence is 30–40%. [16] What is worse, the treatment response rate decreased to 65.8–66.7%, [17, 18] and median survival time is just 14.0–27.2 months. [17, 19] These biological characteristics and the abundant peripheral lymphoid tissue involvement make NPC more prone to the metastasis and invasion compared with the other head and neck tumor types. The recurrence and distant metastasis are causes of treatment failure for NPC patients. Consequently, the majority of patients succumb to the effects of tumor metastasis rather than to the primary lesion. To better promote NPC prognosis and provide a rationale for novel therapies, it was absolutely essential to discover more promising biomarkers of NPC and unravel their underlying molecular mechanisms.

We previously developed a high-ux proteomic classification system to provide the highly accurate and innovative approach for NPC detection and diagnosis. [20] In the recent study, the plasma proteins from 244 NPC patients (II stage: 36 cases; III stage: 104 cases; IV stage: 104 cases) and 104 healthy donors were screened to discover potential novel NPC biomarkers using the techniques of isobaric tags for relative and absolute quantitation (iTRAQ) and liquid chromatography tandem mass spectrometry (LC-MS/MS). These NPC patients did not receive any treatment before diagnosis and were excluded if they had infection, diabetes, hypertension, autoimmune inflammation and other diseases. The findings showed that both of the S100A8 and S100A9 protein levels in the plasma of these NPC patients diagnosed at any different clinical stages were obviously higher than those in healthy donors, which suggested that S100A8/A9 may be the potential plasma biomarkers for NPC diagnosis. [21]

S100A8 (Calgranulin A, MRP8) and S100A9 (Calgranulin B, MRP14) are a pair of calcium binding proteins in S100 protein family, which often form a heterodimer complex in a calcium-dependent manner. They have the amino terminal ef-1 and carboxyl terminal ef-2 hand domains with low molecular weight (Mr 14000 and 13000, respectively) and display important functions in immunity. [22, 23] Tumor microenvironment (TME) is closely related to tumor occurrence and metastasis. The interactions between soluble factors in microenvironment and tumor cells play important roles in cancer development. [24–27] S100A8/A9 is a pair of secreted soluble inflammatory factors. Their main function is to drive a strong chemotaxis effect on the aggregation, adhesion and migration of white blood cells, as well as amplify the local inflammatory effects in microenvironment. [28, 29] To date, the expression of S100A8/A9 proteins in tumor tissues and their roles in microenvironment is still unknown for NPC.

In this study, we would uncover the expression status of S100A8 and S100A9 proteins in NPC tissues and further reveal a molecular basis for their effects on NPC cell proliferation, migration and invasion, which might provide a rationale for NPC prognosis and novel treatment.

2. Materials And Methods

2.1 Paraffin tissue samples

Paraffin embedded tissue samples with detailed pathological diagnosis information were collected from the pathology department of the affiliated first hospital, Guangxi medical university from January 2013 to June 2014 and the pathology department of the affiliated cancer hospital, Guangxi medical university from March 2012 to April 2013. Through careful inquiry of medical record information, only these NPC samples were included considered that the patients have not received any radiotherapy, chemotherapy or targeted therapy before diagnosis, and have no interference of other diseases including
3.1 S100A8 and S100A9 proteins were frequently overexpressed in clinical NPC tissues.

3. Results

3.1 S100A8 and S100A9 proteins were frequently overexpressed in clinical NPC tissues.
To clarify the expression status of S100A8 and S100A9 proteins in NPC tissues, we performed the immunohistochemistry experiments to observe their expressions in 49 NPC cases and 20 chronic pharyngitis (CP) cases. Interestingly, we found a large number of brown-yellow staining signals for S100A8 proteins in the intercellular space and tumor cell cytoplasm of these NPC tissues including phase II, III and IV, while there was only a few brown-yellow staining in CP tissues (Figure. 1A and 1B). Similarly, a large number of brown-yellow staining signals for S100A9 proteins were also detected in the intercellular space and tumor cell cytoplasm of these NPC tissues including phase II, III and IV, while only a few brown-yellow staining for S100A9 was observed in CP tissues (Figure. 1C and 1D). The results indicated that abundant S100A8 and S100A9 proteins were expressed in NPC tissues including II, III and IV clinical stages, mainly distributed in the columnar epithelial interstitium and in the cytoplasm of NPC cells. In contrast, only a few S100A8 and S100A9 proteins existed in the CP tissues and most of them were concentrated in the columnar epithelial interstitium of tissues.

The statistically analyzed results showed that the positive staining area percentage (PSAP) of S100A8 and S100A9 in 49 cases of NPC tissues was 11.74 (8.08, 22.91) and 14.97 (10.55, 21.40), respectively, which were higher than those of 0.29 (0.07, 1.39) and 3.21(1.98, 3.89) in 20 cases of CP tissues, with the significant differences (z-values −6.34 and −5.95, \( P < 0.01 \), respectively) (Figure. 1B and 1D, Table 1).

### Table 1

| Group | N   | S100A8 PSAP(%) | 95% CI       | Z   | P       | S100A9 PSAP(%) | 95% CI       | Z   | P       |
|-------|-----|----------------|--------------|-----|---------|----------------|--------------|-----|---------|
| NPC   | 49  | 11.74(8.08,22.91) | 12.08–17.24 | -6.34 | 0.000   | 14.97(10.55,21.40) | 14.40–19.91 | -5.95 | 0.000   |
| CP    | 20  | 0.29(0.07,1.39)   | 0.30–1.23    |     |         | 3.21(1.98,3.89)   | 2.21–3.62    |     |         |

### 3.2 S100A8 and S100A9 expression levels were closely related to NPC clinical stages.

In further stratified analysis, positive stained area percentages of S100A8 and S100A9 in II, III or IV stage NPC tissues, were both significantly higher than CP tissues (\( P < 0.01 \), respectively) (Figure. 1B and 1D, Table 2). In addition, the positive staining area percentages of S100A8 and S100A9 in advanced stage NPC (III stage or IV stage) were significantly higher than those in early stage NPC (II stage) (\( P < 0.01 \), respectively) (Table 2), while the III stage and IV stage were not statistical different (Figure. 1B and 1D, Table 2).

### Table 2

| G  | N   | S100A8 PSAP(%) | 95% CI       | Z   | P       | S100A9 PSAP(%) | 95% CI       | Z   | P       |
|----|-----|----------------|--------------|-----|---------|----------------|--------------|-----|---------|
|    | 11  | 6.37(3.76,9.36) | 4.46–10.49   | Δ-4.27 | a-3.08 | 5.82(3.24,13.88) | 4.46–10.49 | Δ-3.51 | a-3.64 | a-0.002 | a-0.000 |
|    | 19  | 11.76(9.33,23.47) | 11.60–19.93 | Δ-5.34 | b-0.67 | 18.9(13.25,23.95) | 16.01–23.57 | Δ-5.34 | b-0.51 | b-0.000 | b-0.609 |
|    | 19  | 19.17(10.49,24.56) | 13.25–22.19 | Δ-5.11 | c-3.03 | 15.56(14.32,22.64) | 14.13–24.51 | Δ-5.34 | c-3.34 | c-0.000 | c-0.001 |
| CP | 20  | 0.29(0.07,1.39)   | 0.30–1.23    |     |         | 3.21(1.98,3.89)   | 2.21–3.62    |     |         |

Importantly, we also explored the correlation between positive staining area percentage of S100A8 and S100A9 in NPC tissues and clinical features. Our data suggested that the expression levels of S100A8 and S100A9 in NPC tissues were not related to sex and age, tissue invasion and lymphatic node metastasis, but closely related to clinical stage (\( P < 0.05 \), respectively) (Table 3).
is a potent, selective MEK1/2 inhibitor as controls. The results indicated that AZD6244 treatment (MEK/ERK pathway inhibition) could not diminish the

in medium containing 1 µg/ml S100A8/A9 (p38 MAPK pathway inhibition after SB203580 pretreatment significantly reduced the migration and invasion abilities of these NPC cells even directly cultured

The effect of S100A8/A9 on the migration and invasion ability of NPC cells was tested again by transwell experiments. Importantly, the results showed that

and invasion, and explore whether the p-38 kinase is also involved into this process, a specific p-38 inhibitor SB203580 was used to block p38 MAPK pathway.

Mitogen-activated protein kinase (MAPK) pathway typically responds to extracellular stimulation and is involved into cancer metastasis. S100A8/A9 promote

3.3 S100A8/A9 stimulation promotes NPC cell proliferation, migration and invasion.

We previously found S100A8/A9 proteins were overexpressed in NPC cells and silencing of endogenous S100A8/A9 could significantly reduce NPC cell migration ability. [30] S100A8/A9 as a pair of secreted soluble inflammatory factors was also detected in the intercellular space besides the tumor cell cytoplasm in these NPC tissues as indicated above. To explore the effects of exogenous S100A8/A9 on NPC cell proliferation, we treated the NPC cells with S100A8/A9 at different concentrations to mimic S100A8/A9 inltrated NPC microenvironment and detected cell proliferation by CCK-8 assay. The results suggested that all the three cell lines of CNE1 (high differentiation), CNE2 (low differentiation) and 6-10B (low tumorigenesis and metastasis) have already

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In addition to the cancer cell proliferation, migration and invasion underlying metastatic dissemination is the key clinical problem in NPC. The present clinical investigation indicated NPC prognosis was remarkably associated with S100A8/A9 protein expression abundances. To further explore the effects of S100A8/A9 protein stimulation on NPC migration and invasion, we carried out the transwell migration and invasion experiments using these NPC cell culture models. Interestingly, the results indicated that as low as 1 µg/ml S100A8/A9 added to the lower chamber culture medium could already significant driven

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3.4 S100A8/A9 stimulation promotes migration and invasion of NPC cells via the p-38 MAPK pathway.

Table 3

| Characteristics | N  | S100A8 | B  | S.E | Wald | P     | OR   | 95% CI |
|-----------------|----|--------|----|-----|------|-------|------|--------|
|                 | High | Low |     |     |      |       |      |        |
| Gender          | Male | 38  | 20  | 18  | 1.388 | 1.068 | 1.690 | 0.194  | 4.007 | 0.494 | 32.479 |
|                 | Female | 11 | 7   | 4   |       |       |      |        |
| Age             | < 55 | 40  | 25  | 15  | -2.259 | 1.196 | 3.568 | 0.059  | 0.104 | 0.010 | 1.089 |
|                 | ≥ 55 | 9   | 7   | 2   |       |       |      |        |
| TI              | T1-T2 | 18 | 7   | 11  | -1.700 | 1.317 | 1.665 | 0.197  | 0.183 | 0.014 | 2.416 |
|                 | T3-T4 | 31 | 20  | 11  |       |       |      |        |
| LNM             | NO-N1 | 15 | 4   | 11  | -0.286 | 1.261 | 0.051 | 0.821  | 0.752 | 0.063 | 8.902 |
|                 | N2-N3 | 34 | 23  | 11  |       |       |      |        |
| CS              | I-II | 11 | 1   | 10  | 4.724 | 2.096 | 5.078 | 0.024  | 112.576 | 1.850 | 6850.256 |
|                 | III-IV | 38 | 26  | 12  |       |       |      |        |

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3.3 \text{S100A8/A9 stimulation promotes NPC cell proliferation, migration and invasion.}
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proteins are observed in the intercellular space and tumor cell cytoplasm of these NPC tissues. Interestingly, our results indicated that as low as 0–5 µg/ml S100A8/A9 protein stimulation to mimic the S100A8/A9 infiltrated NPC microenvironment, where the secreted soluble inflammatory factors S100A8/A9 between NPC cancer cells and stromal cells or immune cells including secrete cytokines act important roles in tumorigenesis. [37] Pathological stimulation of nasopharyngeal tissue, caused by bacterial/viral infection or inflammation is a risk factor of NPC. [38] Our previous study has shown that silencing of endogenous S100A8/A9 could obviously inhibit the migration of NPC cells. [39] Overexpressed S100A8/S100A9 proteins have already been observed in a variety of cancer types including breast cancer, [35, 36] prostate cancer, [37, 38] bladder cancer, [39, 40] and colon cancer. [41, 42] S100A8/S100A9 proteins play important roles in promoting cancer proliferation and enhancing their metastasis. Our previous study has shown that silencing of endogenous S100A8/A9 could obviously inhibit the migration of NPC cells. [30] It is well known that pathological stimulation of nasopharyngeal tissue, caused by bacterial/viral infection or inflammation is a risk factor of NPC. [43] The interactions between NPC cancer cells and stromal cells or immune cells including secrete cytokines act important roles in tumorigenesis. [44] Here, we use exogenous S100A8/A9 protein stimulation to mimic the S100A8/A9 infiltrated NPC microenvironment, where the secreted soluble inflammatory factors S100A8/A9 proteins are observed in the intercellular space and tumor cell cytoplasm of these NPC tissues. Interestingly, our results indicated that as low as 0–5 µg/ml

### 3.5 Tumor invasion and migration associated proteins β-catenin and MMP7 were elevated in clinical NPC tissues.

We previously discovered that S100A8 and S100A9 knockdown could significantly reduce the expressions of matrix metalloproteinases (MMPs) in NPC cancer cells[30]. In the present study, we further explored the downstream of signaling pathway and evaluated the β-catenin and MMP7 expression levels in clinical NPC tissues and chronic pharyngitis (CP) tissues, which are two important proteins in tumor cell invasion and migration. Here, the clinical tissues result indicated that abundant β-catenin and MMP7 proteins were expressed in NPC tissues including II, III and IV clinical stages. In contrast, only a few β-catenin and MMP7 proteins existed in the CP tissues (Fig. 4A and B). The statistically analyzed results showed that the positive staining area percentage (PSAP) of β-catenin and MMP7 in 42 cases of NPC tissues was 13.4(9.23,18.52) and 19.6(11.44,26.75), respectively, which were higher than those of 4.65(2.57,7.19) and 0.8(0.32,1.95) in 9 cases of CP tissues, with the significant differences (z-values ~ 3.83 and ~ 4.59, P< 0.01, respectively) (Table 4).

Importantly, the positive stained area percentages of β-catenin and MMP7 in II, III or IV stage NPC tissues, were both significantly higher than CP tissues (P< 0.01, respectively) (Table 4). In further stratified analysis, the positive staining area percentages of β-catenin and MMP7 in advanced stage NPC (III stage or IV stage) were significantly higher than those in early stage NPC (II stage) (P< 0.05, P< 0.01, respectively), while the III stage and IV stage were not statistical different (Table 5).

| Group | N   | β-Catenin PSAP (%) | Z    | P      | MMP7 PSAP (%) | Z     | P      |
|-------|-----|--------------------|------|--------|---------------|------|--------|
| NPC   | 42  | 13.4(9.23,18.52)   | -3.83| 0.000  | 19.6(11.44,26.75) | -4.59| 0.000  |
| CP    | 9   | 4.65(2.57,7.19)    | 0.8(0.32,1.95) |        |               |       |        |

| G | N | β-Catenin PSAP (%) | Z    | P        | MMP7 PSAP (%) | Z    | P        |
|---|---|--------------------|------|----------|---------------|------|----------|
| Ⅰ | 7 | 9.43 (6.84,9.61)    | -1.85| 0.018    | 8.8 (7.54,10.46) | -3.18| 0.000    |
| Ⅱ | 18| 14.98 (8.64,18.52)  | -3.70| 0.000    | 21.44 (14.42,26.86) | -4.12| 0.000    |
| Ⅲ | 17| 15.11 (10.59,25.91) | -3.53| 0.004    | 21.51 (16.76,30.52) | -4.12| 0.000    |
| CP | 9 | 4.65 (2.57,7.19)    | 0.8(0.32,1.95) |        |               |       |        |

4. Discussion

The expression of S100A8/S100A9 and their roles in NPC tissues is still not very clear up till the present moment. Over the past decade, Cheng, et al. identified several proteins including S100A8, S100A9 higher in NPC tissues than in normal nasopharyngeal epithelial tissues (NNET) by mass spectrometry (MS). [32] Li, et al. also independently discovered that the S100A9 protein in NPC was four times higher than that of NNET. [33] Other experiments including western blot were subsequently carried out to provide more evidences that S100A9 may be a potential biomarker in NPC tissue and the S100A9 level is markedly related to clinical typing of NPC. [34] However, it is still unclear that the association between these S100A8/A9 proteins and CP, a high risk stage as the early lesion before NPC, which might affect the specificity of these novel NPC biomarkers. Moreover, the molecular mechanism mediated by S100A8/A9 proteins in NPC migration and invasion is also currently not well understood.

To date, overexpressed S100A8/S100A9 proteins have already been observed in a variety of cancer types including breast cancer, [35, 36] prostate cancer, [37, 38] bladder cancer, [39, 40] and colon cancer. [41, 42] S100A8/S100A9 proteins play important roles in promoting cancer proliferation and enhancing their metastasis. Our previous study has shown that silencing of endogenous S100A8/A9 could obviously inhibit the migration of NPC cells. [30] It is well known that pathological stimulation of nasopharyngeal tissue, caused by bacterial/viral infection or inflammation is a risk factor of NPC. [43] The interactions between NPC cancer cells and stromal cells or immune cells including secrete cytokines act important roles in tumorigenesis. [44] Here, we use exogenous S100A8/A9 protein stimulation to mimic the S100A8/A9 infiltrated NPC microenvironment, where the secreted soluble inflammatory factors S100A8/A9 proteins are observed in the intercellular space and tumor cell cytoplasm of these NPC tissues. Interestingly, our results indicated that as low as 0–5 µg/ml
concentration of S100A8/A9 proteins has already tended to promote NPC cell proliferation. In addition, the migration and invasion abilities were markedly enhanced by as low as 1 µg/ml S100A8/A9 proteins in a variety of NPC cell lines including the low-differentiated CNE2 as well as the high-differentiated CNE1 and even low metastatic 6-10B. Similarly, S100A8/A9 at a relatively low concentration (≤ 25 µg/ml) was reported to promote proliferation, migration and invasion of breast cancer cells. [45] S100A8/A9 proteins as low as 0.4-2 µg/ml were also discovered to promote migration and invasion abilities in one colorectal cancer study. [46] Taken together, it seems clear from present findings that S100A8/A9 protein stimulation could promote proliferation, migration and invasion of NPC cells at a low concentration level.

In addition, we also investigated whether the intracellular pathway is involved in S100A8/A9 stimulated NPC migration and invasion. Our findings indicated that this process might be involved into p38 MAPK pathway. When the p38MAPK pathway was inhibited, the migration and invasion abilities of NPC cells stimulated by S100A8/A9 were diminished. Consistent with the S100A8/A9 overexpression in NPC clinical tissues, the tumor invasion and migration associated proteins β-catenin and MMP7 were also elevated in these clinical NPC tissues. Therefore, here we hypothesized that overexpressed S100A8/A9 as the secreted soluble inflammatory factors in tumor microenvironment might enhance the activity/phosphorylation of p-38 MAPK pathway in cancer cells, which subsequently activated the transcriptional factors and elevated the tumor cell invasion and migration protein expression (e.g. MMP7), and finally promoted NPC cell proliferation, migration and invasion (Figure. 5). As is well known, p38 MAPK pathway is widely involved into cancer growth, development, proliferation, invasion, migration, metastasis, differentiation and other physiological processes. The abnormal or excessive activation of MAPK signaling pathway plays important roles in the malignant transformation and evolution of cells. Of note, similar mechanism was unraveled that activated p38 MAPK pathway under the stimulation of exogenous S100A8/A9 could enhance cell proliferation in breast cancer, [45] or promoted cell migration and invasion in gastric cancer. [31] In addition, clinical tumor metastasis and invasion/migration, is mainly dependent on the activities of protein family-matrix metalloproteinases (MMPs), which is involved into the process of Wnt/β-Catenin and EMT signaling pathway [47].

5. Conclusion

Taken together, our study reveals that S100A8/A9 proteins are highly expressed in NPC tissues, markedly related to NPC clinical stages. Furthermore, S100A8/A9 overexpression in tumor microenvironment could promote NPC migration and invasion via the p38 MAPK signaling pathway and tumor cell invasion and migration protein overexpression (e.g. MMP7). The discovery of secreted soluble inflammatory factors S100A8/A9 as stimulators of NPC migration and invasion as well as better understanding of the S100A8/A9 actions in microenvironment could provide novel clues for NPC diagnosis and therapy.

Abbreviations

| Abbreviation | Description |
|--------------|-------------|
| CP           | chronic pharyngitis |
| iTRAQ        | isobaric tags for relative and absolute quantitation |
| LC-MS/MS     | liquid chromatography tandem mass spectrometry |
| MS           | mass spectrometry |
| TME          | tumor microenvironment |
| TI           | tissue infiltration |
| LNM          | lymph node metastasis |
| CS           | clinical stage |
| IHC          | immunohistochemistry |
| PSAP         | positive staining area percentage |
| MAPK         | mitogen activated protein kinase |
| NNET         | normal nasopharyngeal epithelial tissues |

Declarations

Author Contributions: All authors made a significant contribution to the work reported. In detail, conceptualization, X.Y. and Y.H.; methodology and data analysis, N.X., X.Y., Y.C., Q.H. and Z.K.; writing-original draft preparation, N.X., B.Z., X.Y.,Y.C., Q.H. and Z.K.; writing-review and editing, N.X., B.Z., X.Y. and Y.H.; supervision, Y.H.; project administration, Y.H.; funding acquisition, Y.H.; All authors have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Availability of data and materials: The datasets used and/or analyzed during the current study are available from the corresponding author on the reasonable request.

Ethics approval and consent to participate section:

The reporting studies involving human tissues in the present manuscript have included a statement on ethics approval and consent included the name of the ethics committee that approved the study and the committee's reference number.
Consent for publication section:

The reporting studies involving human tissues have included a statement on ethics approval and consent included the name of the ethics committee that approved the study and the committee's reference number in the present manuscript.

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