**Supplementary materials**

Title: The correlations between CXCL12, CXCR4, EAAT1 and GS in malignant pleural mesothelioma, and CXCL12 regulates cell invasion and migration via CXCR4/EAAT1/GS pathway

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**Supplementary experimental procedures**

**Cell proliferation**

The H2052 cells at a density of 1×104 /mL were seeded in 96-well plates for attachment and were starved in serum-free 1640 medium overnight to synchronize the cell cycle. Then, the cells were subjected to various treatments as indicated in figure. 10 µL MTT (5 mg/mL) was added into each well containing 100 µL cell culture media and incubated at 37°C for 4 h. After that, 200 µL DMSO was added to dissolve the formazan and the optical density was measured at 570 nm wavelength.

**Wound healing analysis**

The H2052 cells at a density of 2×105 /mL were seeded in 24-well plates for confluence and then, the culture plates were scratched linearly with a cell scraper. Subsequently, the cells were subjected to various treatments as indicated in figure. Cells were washed with PBS, fixed in 10% paraformaldehyde (v/v). Photographs were taken in five random fields at 0, 24 and 48 h, respectively and the migratory distance was quantified under light microscopy (100 X magnification).

**siRNA transfections**

Four double-stranded siRNAs targeting the human CXCR4 mRNA (Gene bank No. NM\_001008540.2) and EAAT1 (Gene bank No. NM\_001166696.2) were designed, respectively and synthesized by GenePharma Co., Ltd. (Shanghai, China) along with control siRNAs against CXCR4 and EAAT1, respectively (**Supplementary Table 2**). Briefly, the H2052 cells were seeded in 24-well plates for attachment and then the culture medium was replaced with serum-free 1640 medium. The H2052 cells were transfected with 20 pmol siRNA for 4 – 6 h and then, the culture medium was replaced with 1640 medium containing 10% FBS for 48 h and cell lysates were collected for detection.

**Western blot**

We performed Western blot assay for CXCR4, EAAT1 and GS and analyzed results as described in the **Materials and methods.**

**Real-time PCR (QRT-PCR)**

We performed QRT-PCR for MMP9 and analyzed results as described in the **Materials and methods.**

**Tumor growth in nude mice**

Female BALB/c nude mice, aged 4 - 6 weeks and weighed 16 - 20g were provided by the Experimental Animal Center of the Capital Medical University (CMU) in Beijing and were maintained in the division of laboratory animal science. Forty nude mice were randomly divided into four groups (n = 10): control group (PBS), CXCL12 treatment group, AMD3100 and TFB treatment groups. The H2052 cells were harvested. 0.1 mL of H2052 (with luciferase reporter gene) cells (2×107~3×107/mL) suspension and 0.1 ml of Matrigel were mixed and were inoculated subcutaneously (S.C.) into the back of nude mice. Two weeks later, the mice in control group were injected with PBS solution 0.1 mL/d; The mice in CXCL12 treatment group received 0.1 mL of CXCL12 (20 μg/mL) three times once week; The mice in AMD3100 treatment group received 0.1 mL of AMD3100 (1 mg/mL) once a day; The mice in TFB treatment group received 0.1 mL of TFB (200 nM) three times once week. All treatments (intraperitoneally, i.p.) were processed continuously for three weeks. The luciferin was injected into the nude mice and the fluorescence signal values of tumor were measured weekly, four times in total (2 weeks, 3 weeks, 4 weeks and 5 weeks after H2052 cells implantation). The changes of fluorescence signal values were calculated. At the end of the experiments, the mice were euthanized. All tumors samples from the nude mice were excised and fixed in formalin or frozen in liquid nitrogen for further research. All mouse operations were carried out in accordance with the Institutional Animal Care and Use Committee in CMU (AEEI-2016-162) and comply with the NIH guidelines.

**Statistical analysis**

Statistical analysis was carried out with SPSS 21.0 (SPSS Inc, Chicago, USA) as described in the **Materials and methods.**

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**Figure S1.** Western blot analysis was performed to validate the transfection effects of respective siRNA. H2052 cells were transfected with negtive control siRNA or siRNA targeted to CXCR4 and EAAT1, respectively. Both the targeted CXCR4 and EAAT1 were downregulated. 1, 2, 3, 4 represents siRNA1, siRNA2, siRNA3, siRNA4 targeted to CXCR4 and EAAT1, respectively.

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**Figure** S**2.** Effect of CXCL12 on the viability in MPM cell line. H2052 cells were treated with various concentrations of CXCL12 (0 - 100 ng/mL) (A) or AMD3100 (0 - 10 µM) (C) for 24 h and morphological images in vitro from different treatment groups were shown. (B), The cell viability was evaluated by MTT assay. \* p < 0.05 versus control, as indicated. The data were expressed as mean ± SD from quadruplicates and repeated three times independently.

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**Figure** S**3.** Effects of CXCL12 on the migration in MPM cell line. (A), H2052 cells were treated with various concentrations of CXCL12 (0 - 100 ng/mL) for 24 h, 48 h and subsequently the cells migration were examined by wound healing assay. Representative images (upper panal) and the corresponding quantitative analysis (lower panal) from different groups were shown. (C), H2052 cells were pretreated with AMD3100 for 30 min and then treated with CXCL12 (100 ng/mL) for 24 h and 48 h. The cells migration were examined by wound healing assay. Representative images (upper panal) and the corresponding quantitative analysis (lower panal) from different groups were pesented. Values were expressed as relative migration distance (relative ratio of different treatment groups to untreated control). \* p < 0.05 versus control. # p < 0.05 versus CXCL12 treatment, as indicated. The data were expressed as mean ± SD from repeated three independent experiments.

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**Figure S4.** Effect of CXCL12 on the MMP9 mRNA level in MPM cell line. H2052 cells were treated with or without CXCL12 (100 ng/mL) for 5 min and the MMP9 mRNA was determined by QPCR. Values were expressed as fold of untreated control levels. The data shown were representative of three independent experiments.

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**Figure S5**. Effect of CXCL12 on tumor growth in nude mice. 2 × 106 H2052 cells were inoculated subcutaneously (S.C.) into the left dorsal of nude mice, and the mice were treated with AMD3100, TFB, CXCL12 or vehicle starting 2 weeks after implantation, as described in **Materials and methods**. Obvious tumor appearance and (A) and bioluminescence/fluorescence signal value (B) could be visible on the mouse back.

Table S1. Primer pairs of CXCR4, GS, EAAT1, MMP9 and GAPDH

|  |  |  |
| --- | --- | --- |
| Target gene | Primer pairs | |
| Forward primer (5’ - 3’) | Reverse primer (5’ - 3’) |
| CXCR4 | TCAgTCTggACCgCTACCTg | gggATCCAgACgCCAACATA |
| GS | CAATCgAAggCCTgCAgAgA | ATACTCCTgCTCCATgCCAA |
| EAAT1 | TTgCTgCAAgCACTCATCAC | gCTTgTCCACgCCATTgTTC |
| MMP9 | TGTACCGCTATGGTTACACTCG | GGCAGGGACAGTTGCTTCT |
| GAPDH | CTTCTTTTgCgTCgCCAgCC | ggCgCCCAATACgACCAAA |

Table S2. CXCR4 and EAAT1 siRNA oligonucleotide sequences

|  |  |  |
| --- | --- | --- |
| Gene | SiRNA oligos (5’-3’) | |
| Sense | Anti-sense |
| CXCR4(1) | GGCUGAAAAGGUGGUCUAUTT | AUAGACCACCUUUUCAGCCTT |
| CXCR4(2) | GCCUUACUACAUUGGGAUCTT | GAUCCCAAUGUAGUAAGGCTT |
| CXCR4(3) | CAAGCAAGGGUGUGAGUUUTT | AAACUCACACCCUUGCUUGTT |
| CXCR4(4) | GCACAAGUGGAUUUCCAUCTT | GAUGGAAAUCCACUUGUGCTT |
| CXCR Control | ACCAGGUUAUUGCAGUACGTT | CGUACUGCAAUAACCUGGUTT |
| EAAT1(1) | CGGGGAAUAUUAUCAGAUGTT | CAUCUGAUAAUAUUCCCCGTT |
| EAAT1(2) | CCAUAACCAGCUAUACCUUTT | AAGGUAUAGCUGGUUAUGGTT |
| EAAT1(3) | CCAAGAAGAAAGUGCAGAATT | UUCUGCACUUUCUUCUUGGTT |
| EAAT1(4) | CACUGAAGUGCAAAGAAGATT | UCUUCUUUGCACUUCAGUGTT |
| EAAT1  Control | ACCAGGUUAUUGCAGUACGTT | CGUACUGCAAUAACCUGGUTT |

Table S3. The changes of fluorescence signal values of four groups

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| After starting of treatments | Control | CXCL12 | AMD3100 | TFB |
| 1 week | 0.98 ± 1.46 | 1.31 ± 1.31 | -0.16 ± 0.97 | 0.75 ± 1.23 |
| 2 weeks | 2.80 ± 3.20 | 58.44 ± 63.82 | 2.13 ± 3.50 | 3.82 ± 3.24 |
| 3 weeks | 6.89 ± 3.26 | 43.00 ± 36.58 | 0.97 ± 0.82 | 2.35 ± 2.49 |

Table S4. p values between groups at 3 weeks after starting of treatments

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | AMD3100 | Control | CXCL12 | TFB |
| AMD3100 |  | P = 0.0021 | P = 0.0002 | P > 0.05 |
| Control |  |  | P = 0.0368 | P = 0.0157 |
| CXCL12 |  |  |  | P = 0.0014 |
| TFB |  |  |  |  |