Title	子宮体部漿液性癌のドライバー遺伝子STAT1を標的とする新規治療法の開発
Sub Title	Development of novel therapeutic methods targeting the driver gene STAT1 of serous papillary endometrial cancer
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Abstract	子宮体部漿液性癌は予後が悪く, 化学療法に対する耐性も高い。再発率もおよそ40%と示されている。本研究は, 漿液性腺癌のSTAT1遺伝子発現を中心とした癌化学療法耐性機序を解明した。 STAT1のセリンリン酸化が中心的役割を果たしており, 高STAT1発現がDNA損傷抵抗の増強や, プラチナ細胞内蓄積濃度の減少と関連していることが分かった。TBBを加えると, STAT1のセリンが抑制でき, プラチナ感受性が増強した。さらに, 動物実験で同様な感受性増強結果を認めた。以上より, STAT1を標的治療ターゲットとして阻害すると, 子宮内膜漿液性腺癌のプラチナ耐性克服の可能性が示唆された。 Endometrial cancer is known to have a relatively good prognostic factors among gynecologic malignancies ; however, advanced progressive type, Serous Papillary Endometrial Cancer (SPEC), possesses chemo-refractory feature with poor prognosis and 40% recurrence rate of cases. In this study, we revealed STAT1 gene expression play an important role in SPEC chemo-resistance to Cisplatin treatment. Serine phosphorylated STAT1 was a major regulator of STAT1 activity in SPEC chemo-resistance feature. Constitutively high expression of STAT1 attenuates the tumor DNA damage, tumor cytoplasmic accumulation and activity. We showed that STAT1 inhibition by TBB pre-treatment increased SPEC chemo-sensitivity to Cisplatin in vitro. TBB pre-treatment followed by Cisplatin has been proved to be effective in eradicating SPEC tumor in mouse xenograft model. Therefore, we proposed STAT1 as a novel potential molecular targeted therapy to overcoming SPEC chemo-resistance to Cisplatin.
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研究課題名（和文）子宮体部漿液性癌のドライバー遺伝子STAT1を標的とする新規治療法の開発

研究課題名（英文）Development of novel therapeutic methods targeting the driver gene STAT1 of serous papillary endometrial cancer

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研究成果の概要（和文）：子宮体部漿液性癌は予後が悪く、化学療法に対する耐性も高い。再発率もおよそ４０％と示されている。本研究は、漿液性腺癌のSTAT1遺伝子発現を中心とした癌化学療法耐性機序を解明した。STAT1のセリンリン酸化が中心的役割を果たしており、高STAT1発現がDNA損傷抵抗の増強や、プラチナ細胞内蓄積濃度の減少と関連していることが分かった。TBBを加えると、STAT1のセリンが抑制でき、プラチナ感受性が増強した。さらに、動物実験で同様な感受性増強結果を認めた。以上より、STAT1を標的治療ターゲットとして阻害すると、子宮内膜漿液性腺癌のプラチナ耐性克服の可能性が示唆された。

研究成果の概要（英文）：Endometrial cancer is known to have a relatively good prognostic factors among gynecologic malignancies; however, advanced progressive type, Serous Papillary Endometrial Cancer (SPEC), possesses chemo-refractory feature with poor prognosis and 40% recurrence rate of cases.
In this study, we revealed STAT1 gene expression play an important role in SPEC chemo-resistance to Cisplatin treatment. Serine phosphorylated STAT1 was a major regulator of STAT1 activity in SPEC chemo-resistance feature. Constitutively high expression of STAT1 attenuates the tumor DNA damage, tumor cytoplasmic accumulation and activity. We showed that STAT1 inhibition by TBB pre-treatment increased SPEC chemo-sensitivity to Cisplatin in vitro. TBB pre-treatment followed by Cisplatin has been proved to be effective in eradicating SPEC tumor in mouse xenograft model. Therefore, we proposed STAT1 as a novel potential molecular targeted therapy to overcoming SPEC chemo-resistance to Cisplatin.

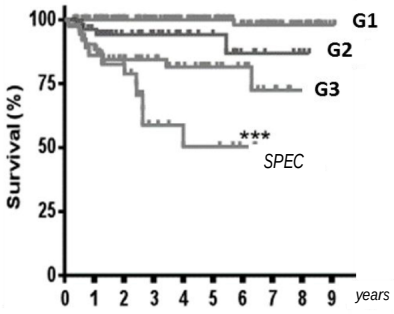
研究分野：　Tumor Biology

キーワード：　Endometrial cancer　Serous papillary　STAT1　chemo-resistance

１．研究開始当初の背景

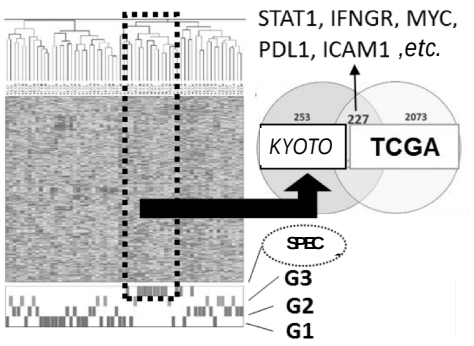
Endometrial cancer is known to have a relatively good prognostic factors among gynecologic malignancies; however, advanced progressive type possesses high recurrence risk and malignant pathological features. In particular, serous subtype, Serous Papillary Endometrial Cancer (SPEC), has a high metastatic potential and resistant to chemotherapy. SPEC has a poor prognosis with recurrence rate around 40% of cases (Fig. 1A, Cancer Res., 2014, Int J Cancer, 2013).

Fig. 1A. Overall survival of Kyoto Endometrial Cancer (n=63 cases)



Recently, integrated genomic analysis of chemo-refractory cancer attempt to elucidate the appropriate therapeutics drugs for better clinical management. The Cancer Genome Atlas (TCGA) project has been performed and classified endometrial carcinoma into 4 categories by integrated genomic analysis. Serous papillary endometrial carcinoma was characterized by its malignant features which is accompanied by TP53 and PIK3CA mutation (Nat. Genet. 2012; Nature. 2013). However, the specific responsible pathways are still remained unknown. Therefore, our in-silico analysis revealed 227 SPEC specific genes signature which were shared between our cohort and TCGA cohort. Those SPEC genes signature are mainly related to the STAT1 pathway (Fig. 1B, Cancer Res., 2014).

Fig. 1B. Specific genes signature of Serous Papillary Endometrial Cancer (SPEC)



We are going to elucidate the relationship between STAT1 pathway activity, and its potential roles in progressive features of SPEC. Moreover, we are going to reveal the potential roles of STAT1 in the mechanism of anticancer drug resistance possessed by SPEC tumor cell. In this study, we investigated mechanisms of tolerance to various anticancer drugs using different serous papillary endometrial carcinoma cell lines with different expression level of STAT1. We clarified the roles of STAT1 on the anticancer drug resistance mechanism of serous papillary endometrial cancer by establishing individual treatment of each serous papillary endometrial cancer cell line with STAT1 inhibitor.

The effectiveness of STAT1 inhibitor on serous papillary endometrial cancer were shown by functional inhibition potential of this agent on the progressive features of STAT1 overexpressed serous papillary endometrial cancer cell lines. We also examined the dynamic changes of drug resistance trait and cancer stemness in *in-vitro* culture system. Moreover, we examined the effectiveness of STAT1 inhibitor *in-vivo* by administering it into the tumor xenograft on mice and observe the potential inhibition of tumor growth in mice. These functional experiments revealed the potential therapeutic effects of STAT1 inhibitor in combination with anti-cancer drugs in chemo-refractory serous papillary endometrial cancer management.

２．研究の目的

The main purpose of this study is:
「Development of novel therapeutic methods targeting the driver gene STAT1 of serous papillary endometrial carcinoma」.

Serous papillary endometrial cancer is featured by its high metastatic potential, chemotherapy resistance and its worst prognosis among endometrial cancer subtypes. This study aims to:

(1) Clarify STAT1 as a driver gene which contribute to chemotherapy resistance in serous papillary endometrial cancer.
(2) Elucidate STAT1 targeted therapy to overcoming chemotherapy resistance in serous papillary endometrial cancer.
(3) Develop novel therapeutic method targeting STAT1 in order to develop better clinical management of serous papillary endometrial cancer.

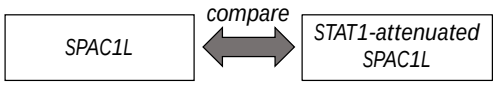
3．研究の方法

This study was divided into two stages of experiments as below.

(1) Investigation the mechanisms by which STAT1 expression leads to platinum resistance in serous papillary endometrial cancer.

We utilized SPEC cell line, SPAC1L, which is highly expressing STAT1 gene. Since SPAC1L shows resistance to irinotecan, we examined the possibility of involvement of DNA damage mechanism in SPAC1L chemo resistance. We developed STAT1-knocked down SPAC1L cell line. DNA repair mechanism was compared between these 2 cell lines by immunostaining and flowcytometry of DNA damage marker as describe previously (Murai, Mol Cell Biol, 2011) (Fig. 2A).

Fig 2A. Examination on how STAT1 expression is associated with platinum chemoresistance.



- *DNA damage/repair marker gene: rH2AX*
- *Other DNA damage/repair molecules*
- *DNA damage/repair genes signatures within Kyoto cohort, TCGA, GSE17025,etc.*

Furthermore, we also performed microarray analysis of both cell lines. In silico analysis of DNA damage/repair signatures was carried out. DNA damage/repair activity in human SPEC tissue was examined based on the signatures obtained from cell lines microarray analysis (Fig.2B).

STAT1 activity and cellular function is controlled by its phosphorylation. We also investigated in which phosphorylation site, Ser or Tyr, is involved in the malignant features of SPEC, using SPAC1L and other SPEC cell lines.

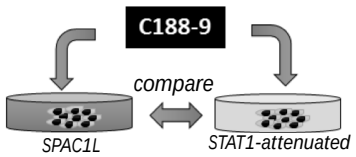
(2) Investigation of therapeutic effect of the novel STAT1 inhibitor C-188-9 against serous papillary endometrial cancer.

At first, we performed *in-vitro* functional study of STAT inhibitor using SPAC1L and its STAT1-attenuated counterparts. A STAT1 inhibitor, C-188-9, was administered in cell culture system, and various functional experiments (i.e. proliferation assay, migration assay, etc.) were performed to revealed the therapeutic effect of C-188-9 compound in SPEC (Fig.2B-1).

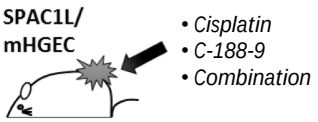
Next step, we performed *in-vivo* experiment using tumor xenograft system in immune-deficient mice. SPAC1L and its STAT1-attenuated counterparts cell lines were inoculated subcutaneously to the immune-deficient mice. After xenograft tumor reached 5 mm^2 in size, C-188-9 (50 ng/g BW, once daily intraperitoneally, 5 days) and cisplatin (doses as describe previously), was administered to the mice, either as single agent or in combination. Therapeutics effects were defined by measuring mouse body weight, tumor size, and safety of the drugs (Fig. 2B-2).

Fig 2B. Examination on how STAT1 expression is associated with platinum chemoresistance.

1. In-vitro functional assay



2. In-vivo tumor xenograft model

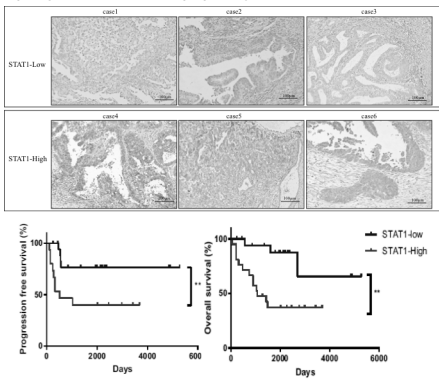


4．研究成果

(1) STAT1 expression level is associated with serous papillary endometrial cancer prognosis.

It has been shown already in our previous study that STAT1 expression is constitutive high in serous papillary endometrial cancer. In this study, we revealed that STAT1 expression is associated with prognosis within serous papillary endometrial cancer itself (Fig. 3). This finding suggests that STAT1 may

Fig 3. Association of STAT1 expression and prognosis of serous papillary endometrial cancer

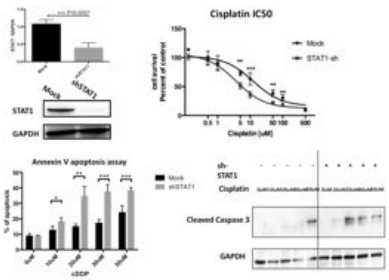


be potential to be targeted for therapeutics purposes.

(2) Suppression of STAT1 expression increase chemo-sensitivity of serous papillary endometrial cancer cell lines to Cisplatin treatment.

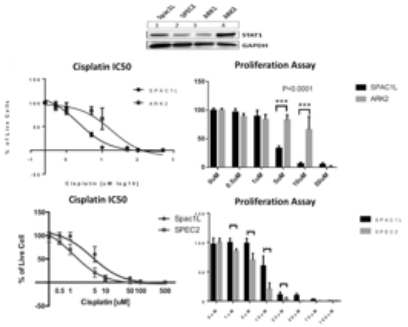
We established STAT1 knocked-out SPAC1L cell by using STAT1-shRNA transduction system. We confirmed the successful suppression rate of STAT1 in mRNA and protein level. Chemo-sensitivity of SPAC1L cell to Cisplatin was increased by STAT1 suppression. This finding was confirmed by increasing of apoptosis rate of STAT1 knocked-out SPAC1L under Cisplatin treatment in dose-dependent manner (Fig.4A).

Fig 4A. STAT1 suppression increase Cisplatin chemo-sensitivity of SPAC1L



Furthermore, we also performed the Cisplatin IC50 assay and cell proliferation assay of several serous papillary endometrial cancer cell lines with different status of STAT1 expression. ARK2 cell represented highest STAT1 expression constitutively, SPAC1L cell represented intermediate STAT1 expression, while SPEC2 represented lowest STAT1 expression within SPEC cell lines. In line with above results, chemo-sensitivity of SPEC cells were correlated with their STAT1 expression (Fig.4B).
These findings suggest that STAT1 might play an important role in SPEC chemo-resistance feature.

Fig 4B. Chemo-sensitivity of SPEC cell lines are related to STAT1 expression



(3) STAT1 play an important role in SPEC chemo-resistance possibly through DNA damage/repair mechanism and Cisplatin activity.

Next, we examined the possible mechanism through which STAT1 suppression can play roles in increasing SPEC chemo-sensitivity.

One of the classic phenomenon in chemotherapy is the dynamic of DNA damage and repair pathway. We performed immunofluorescence staining of DNA damage marker, gamma-H2AX, to see the dynamic of DNA repair as a response of cells to chemo-therapy stress.

Fig 5A. STAT1 suppression increased SPEC cells chemo-sensitivity through DNA damage/repair mechanism

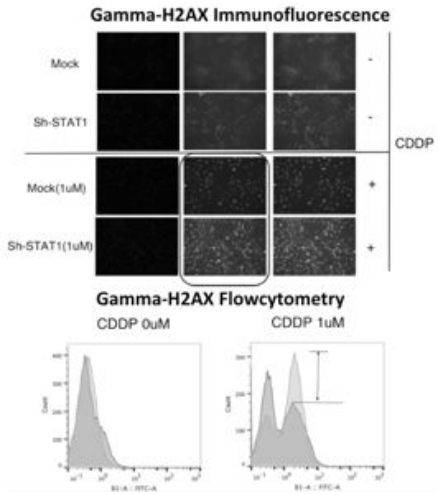
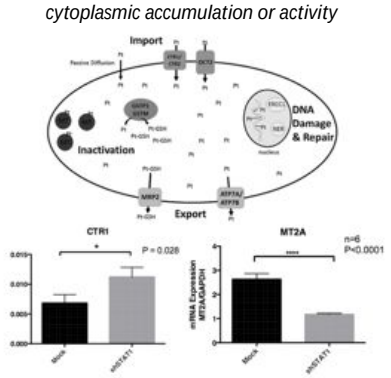


Figure 5A. showed the increasing of gamma-H2AX in SPAC1L STAT1 knocked-out cells which suggests increasing of DNA damage under Cisplatin treatment. This finding was confirmed by gamma-H2AX flowcytometry assay.

Since Cisplatin therapeutics effect was also related with the kinetic of Cisplatin itself, therefore we also observed the kinetic of Cisplatin by examining Cisplatin cytoplasmic accumulation marker status, CTR1; and Cisplatin inactivation marker status, MT2A.

Fig 5B. STAT1 may attenuate Cisplatin cytoplasmic accumulation or activity



As shown in figure 5B, suppression of STAT1

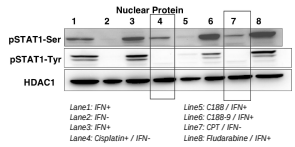
increased accumulation of cytoplasmic Cisplatin by increasing CTR1 marker gene. At the same time, STAT1 suppression also decreasing Cisplatin inactivation by suppressing MT2A marker gene. These findings addressed that STAT1 also play an important role in Cisplatin kinetic in SPEC.

(4) Serine phosphorylation of STAT1 as a major regulator in SPEC chemo-resistance to Cisplatin treatment.

The active form of STAT1 in its signaling pathways is phosphorylated STAT1. STAT1 can be effectively functional in signaling pathway through 2 phosphorylation site, Serine and Tyrosine.

We performed western blotting staining of STAT1 phosphorylation status under Cisplatin treatment of SPAC1L STAT1 knocked-out and its counterpart. We revealed serine phosphorylated STAT1 seems to be a major regulator of Cisplatin chemo-resistance in serous papillary endometrial cancers (Fig. 6).

Fig 6. Serine phosphorylation of STAT1 seems to be a major regulator in chemo-resistance



(5) STAT1 targeted therapy is potential to overcoming Cisplatin chemo-resistance in serous papillary endometrial cancer.

Our previous results above have addressed STAT1 as an important regulator of Cisplatin chemo-resistance in serous papillary endometrial cancer cell lines *in vitro*, which suggest STAT1 targeted therapy might be potential to be applied in serous papillary endometrial carcinoma treatment. To bridging those *in vitro* findings and the potential clinical efficacy, we performed tumor xenograft *in vivo* model to show the potential efficacy of STAT1 targeted therapy using STAT1 inhibitor.

Our collaborator Dr. David. J. Tweardy from MD Anderson Cancer Center has invented C-188-9 as a small molecule targeting STAT3 in various tumors. Since STAT1 and STAT3 are closely correlated in molecular function, we were planning to utilize this compound as STAT1 inhibitor, supported by Dr. Tweardy data which showed C-188-9 was also functioned as STAT1 inhibitor. Unfortunately, during our *in vitro* screening, C-188-9 did not show an inhibition potential towards STAT1. Moreover, we found another compound, TBB, a CK2 (upstream regulator of STAT1) inhibitor which is potential in inhibiting STAT1 as a downstream of CK2.

Fig 7A. Efficacy of TBB, a CK2 inhibitor, in increasing SPAC1L chemo-sensitivity to Cisplatin

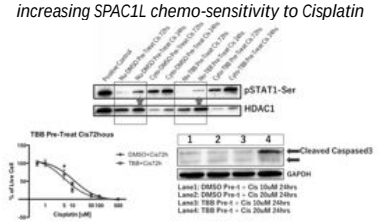
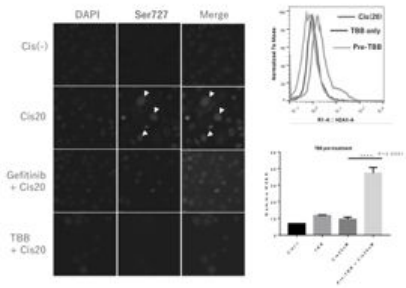


Figure 7A showed the inhibition effect of TBB on STAT1 serine phosphorylation in time dependent manner. Pre-treatment of TBB prior Cisplatin also increased the chemo-sensitivity of SPAC1L to Cisplatin as shown by decreasing of Cisplatin IC50 dose after TBB pre-treatment. Apoptosis of SPAC1L were increased by TBB pre-treatment followed by Cisplatin.

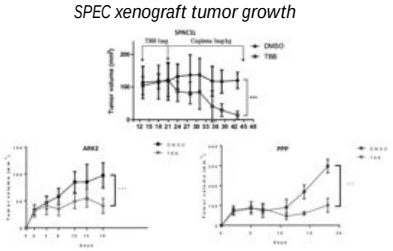
To confirm the molecular mechanism by which TBB increased SPAC1L chemo-sensitivity, we performed DNA damage/ repair marker immunofluorescence staining.

Fig 7B. TBB, a CK2 inhibitor, increased DNA damage/repair pathway in SPAC1L



As shown in figure 7B., TBB pre-treatment followed by Cisplatin increased the DNA damage/repair activity compared to Cisplatin treatment only. TBB pre-treatment also inhibit the expression of serine phosphorylated STAT1 (as a response of Cisplatin chemotherapy).

Fig 7C. TBB has a potential efficacy in inhibiting SPEC xenograft tumor growth



Furthermore, we utilized SPAC1L, ARK2,

and mouse serous papillary endometrial cancer cell line, PPP (developed by our collaborator, Dr.Daikoku), to generate tumor xenograft mouse model. Figure 7C showed the efficacy of TBB pre-treatment followed by Cisplatin *in vivo* compared to single Cisplatin treatment in tumor xenograft models.

Taken together, our study suggested STAT1 as a novel potential molecular targeted therapy to overcoming SPEC chemo-resistance to conventional molecular targeting drugs, such as Cisplatin.

５．主な発表論文等
（研究代表者、研究分担者及び連携研究者には下線）

〔学会発表〕（計 2 件）

(1).「STAT1 Phosphorylation May Confer Cisplatin Resistance in Serous Papillary Endometrial Cancer」
Xiang Zeng, Tsukasa Baba, Budiman Kharma, Noriomi Matsumura, Kaoru Abiko, Junzo Hamanishi, Ken Yamaguchi, Ikuo Konishi.
English oral presentation at The 75th Annual Meeting of the Japanese Cancer Association, Yokohama, October 6th-8th, 2016

(2) 「STAT1 Phosphorylation May Confer Cisplatin Resistance in Serous Papillary Endometrial Cancer」
Xiang Zeng, Tsukasa Baba, Budiman Kharma, Noriomi Matsumura, Kaoru Abiko, Junzo Hamanishi, Ken Yamaguchi, Ikuo Konishi.
Japanese oral presentation at The 76th Annual Meeting of the Japanese Cancer Association, Yokohama, September 28th-30th, 2017

６．研究組織

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