(micronuclei) by flow cytometry of human reticulocytes. FASEB J 19:485–487.

Phonethepswath S, Bryce SM, Bemis JC, Dertinger SD. 2008. Erythrocyte-based Pig-a gene mutation assay: Demonstration of cross-species potential. Mutat Res 657:122–126.

Phonethepswath S, Franklin D, Torous DK, Bryce SM, Bemis JC, Raja S, Avlasevich S, Weller P, Hyrien O, Palis J, MacGregor JT, Dertinger SD. 2010. Pig-a mutation: Kinetics in rat erythrocytes following exposure to five prototypical mutagens. Toxicol Sci 114:59–70.

Rothfuss A, Honma M, Czich A, Aardema MJ, Burlinson B, Galloway S, Hamada S, Kirkland D, Heflich RH, Howe J, Nakajima M, O'Donovan M, Plappert-Helbig U, Priestley C, Recio L, Schuler M, Uno Y, Martus H-J. 2011. Improvement of in vivo genotoxicity assessment: Combination of acute tests and integration into standard toxicity testing. Mutat Res 723:108–120.

Thybaud V, Dean S, Nohmi T, de Boer J, Douglas GR, Glickman BW, Gorelick NJ, Heddle JA, Heflich RH, Lambert I, Martus H-J, Mirsalis JC, Suzuki T, Yajima N. 2003. In vivo transgenic mutation assays. Mutat Res 540:141–151.

キーワード解説

Glial L-glutamate transporters

# グリア型グルタミン酸トランスポーター

佐藤 薫

## 1. グリア型グルタミン酸トランスポーター

グルタミン酸（L-glu）は，ほ乳類中枢神経系において高次神経機能を担う重要な興奮性神経伝達物質である．しかし一方で，細胞外に高濃度のグルタミン酸が存在すると，興奮毒性によって神経細胞は死に至る．グリア細胞に存在するL-gluトランスポーターはシナプス終末から放出されたL-gluを速やかに取り込み，正常なシナプス伝達環境を整えている．これらの機構が破綻し，細胞外L-glu濃度が上昇すると神経細胞は障害をうけてしまう．

## 2. 構造と機能

中枢神経系において細胞膜に存在するL-gluトランスポーターはこれまでにEAAT1～5の5つのサブタイプが同定されているが，このうちEAAT1（齧歯類ではGLAST）とEAAT2（同じくGLT1）がグリア細胞（特にアストロサイト）に存在する．EAAT1は中枢神経系に広範に分布し，小脳バーグマングリアに高発現している．EAAT2は中枢神経系のグリア細胞に高発現しており，シナプス伝達で放出されたL-gluの90%を取り込むとされている．細胞内にN末端とC末端があり，6～8個の膜貫通部位，1～2個の膜を完全に貫通していないループ構造を持つと考えられているが，詳細な構造については長らく論争が続いていた．最近では，古細菌のEAAT homolog，Glt（Ph）のX線結晶解析により詳細な解析が進められている(1)．高次構造として3量体を形成することが知られているが，Glt（Ph）の解析により，3量体構造でTM7, 8，HP1, 2ドメインが基質の輸送に関与していることが明らかとなった(2)．EAATsのL-glu取り込みメカニズムは，$Na^+$ 2～3分子の結合，L-glu 1分子の結合，$H^+$ 1分子の結合，L-glu細胞内取り込みに連動した$Na^+$，$H^+$の流入，細胞外への1分子の$K^+$流出，というのが一連のサイクルと考えられている．Glt（Ph）の解析から，C末端がL-glu取り込みkineticsを調節していること(3)，HP2 loopが基質結合のattractorであること(4)が示されている．また，$Na^+$を取り込むL-gluトランスポーターと$Na^+K^+$-ATPaseが複合体として機能している点は細胞機能を考える上で興味深い(5)．これらのイオンの動きとは独立して$Cl^-$流入が起こる．このような二重の役割をもつトランスポーターとしては，シナプス前部位に存在するドパミントランスポーターが$Cl^-$コンダクタンスにより興奮性を調節するという現象が有名であるが(6)，グリア型L-gluトランスポーターは$Cl^-$コンダクタンスが低いため(7) 同様の機能は考えにくく，その役割は明らかでない．L-gluトランスポーター調節機構としては特に各種キナーゼ，scaffolding protein等によるtrafficking制御について研究が進んでいる．Glu transporter-associated proteins（GTRAPs）やAjubaといったscaffolding protein(8, 9)はアクチン動態とも相関があり，細胞骨格の再構成が起こりやすいグリア細胞でのL-gluトランスポーターtrafficking制御との関連に興味が持たれる．

## 3. 創薬標的としての可能性

L-gluトランスポーター機能を増強すれば，興奮毒性の関与が大きい中枢神経疾患に治療効果を発揮するのではないか，と期待されている．たとえば，キナーゼを介してL-glu取り込みを上昇させるMS-153が動物虚血モデルにおいて効果を示している(10)．筋萎縮性脊索硬化症（amyotrophic lateral sclerosis: ALS）はL-gluトランスポーターを標的とした創薬が最も期待されている中枢神経疾患といえる．そもそも，EAAT2自身が病因と深く関わっていることも示唆されている．Superoxide dismutase 1変異ALSモデルマウスにおいて，EAAT2スプライシング異常が報告されている(11)．同様の異常はヒトALS患者脳でも報告されているが，EAAT2スプライシング異常により正常のEAAT2発現は阻害されてしまう(12)．Neuronの変性がアストロサイトのKBBPプロモーター活性を低下させ，EAAT2の発現低下，病態増悪につながることも報

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告されている(13). RothsteinのグループはFDAで認可済みの薬物をスクリーニングしβ-lactam系の抗生物質がEAAT2の発現を選択的に上昇させることを見いだした. そのうち, ceftriaxonはNF-κB pathwayを介してEAAT2発現を上昇させるが(14), ALS動物モデルで作用を発揮し, 米国ではすでに臨床研究phase Ⅲに至っている(15). 一方で, L-gluトランスポーター機能を減弱することにより治療効果が期待されている中枢神経疾患もある. 統合失調症ではL-gluトランスポーターの発現が上昇しており, クロザピンのような非定型抗精神病薬はこの発現上昇を阻害することが作用機序の1つであると考えられている(16). 実際, 統合失調症患者の視床下部においてEAAT1, EAAT2の発現上昇が報告されているが(17), これに対しては反論も多い. 患者脳の場合, 薬歴等の影響が大きく, 統合失調症では陽性症状と陰性症状の間で発症メカニズムが異なる可能性も示唆されており, 発現量変化からL-gluトランスポーターと発症との関連を議論するのが難しい一面もある. 動物実験では, ceftriaxonによってGLT1の発現が上昇するとprepulse inhibition(直前の音刺激により次の音刺激に対する反応が減弱する現象. 統合失調で異常が現れるとされる)で異常が現れる(18). 統合失調症モデル動物ではGLASTの発現が上昇しており, L-gluトランスポーター阻害薬が奏功している(19). 一方で, GLT1, GLASTの発現量が低い変異マウスでは統合失調症の陰性症状によく似た行動が現れることも報告されていることから(20), 統合失調症ではやはり陽性症状と陰性症状とを切り分けて創薬標的を見いだすことが重要かもしれない. そのための有効なツールとして血液脳関門を通過するL-gluトランスポーター阻害薬の開発が期待される.

## 4. 炎症との関わり

上記以外でも, 大うつ病, アルツハイマー病など, L-gluトランスポーターの変調が報告されている中枢神経疾患は多い. これらの疾患には全て炎症が伴っていることから, 我々は炎症がアストロサイトL-gluトランスポーターの機能を損なわせているのではないかと考えている. アストロサイト, ミクログリア, ニューロンからなる培養系をLPS処置することによりin vivoに近い炎症を再現してみると, ミクログリアからL-glu放出がおこり, その結果生じた細胞外L-glu濃度の上昇に長時間さらされることによってアストロサイトL-gluトランスポーターの発現量が低下することを見いだした(投稿準備中). このメカニズムについては現在検討中であるが, 病態時のL-gluトランスポーターの挙動がグリア細胞間のコミュニケーションによって調節されていることを示唆しており興味が持たれる.

## おわりに

L-gluトランスポーターの変調を伴う中枢神経疾患の種類は実は大変な数に上るが, その変調が病因か結果か, という問いに対する明確な答えを得るにはもう少し時間がかかりそうである. また, 病態におけるL-gluトランスポーターの機能変化を明らかにすることは, 病態時の中枢神経細胞間のコミュニケーションを解明することにもつながり, あらたな創薬アプローチを提示することになるだろう.

## 文　献


1) Ryan RM, et al. Nat Struct Mol Biol. 2007;14:365-371.
2) Leighton BH, et al. J Biol Chem. 2006;281:29788-29796.
3) Leinenweber A, et al. J Biol Chem. 2011;286:1927-1937.
4) Shrivastava IH, et al. J Biol Chem. 2008;283:28680-28690.
5) Rose EM, et al. J Neurosci. 2009;29:8143-8155.
6) Mortensen OV, et al. Eur J Pharmacol. 2003;479:159-170.
7) Grewer C, et al. J Membr Biol. 2005;203:1-20.
8) Lin CI, et al. Nature. 2001;410:84-88.
9) Marie H, et al. Mol Cell Neurosci. 2002;19:152-164.
10) ShimadaF, et al. Eur J Pharmacol. 1999;386:263-270.
11) Munch C, et al. J Neurochem. 2002;82:594-603.
12) Maragakis NJ, et al. Ann Neurol. 2004;55:469-477.
13) Yang Y, et al. Neuron. 2009;61:880-894.
14) Lee SG, et al. J Biol Chem. 2008;283:13116-13123.
15) Rothstein JD, et al. Nature. 2005;433:73-77.
16) Vallejo-Illarramendi A, et al. Glia. 2005;50:276-279.
17) Smith RE, et al. Am J Psychiatry. 2001;158:1393-1399.
18) Bellesi M, et al. Glia. 2009;57:703-713.
19) Lu L, et al. Behav Brain Res. 2011;220:132-139.
20) Tanaka K. Nihon Shinkei Seishin Yakurigaku Zasshi. 2009;29:161-164.

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# Ephrin/Eph 受容体シグナルを介した neuron-glia communication

佐藤 薫

中枢神経系のシナプス機能において neuron-glia communication が重要であることがわかってきている．Glia の中でも特に astrocyte によるシナプス機能調節については精力的に研究が進められており，シナプス伝達の際に gliotransmitter と呼ばれる伝達物質（グルタミン酸［L-Glu］や ATP）を放出し neuron の興奮性やシナプス伝達を調節することが報告されている(1, 2)．

しかし最近，neuron-glia communication はこのような液性因子を介したものにとどまらないことが明らかとなってきた．

Neuron の樹状突起には spine とよばれる棘状の構造が無数に存在し，シナプスの入力を受けている．この spine がシナプス周囲アストロサイトの突起とダイナミックに接触を繰り返す様子がタイムラプスイメージングで捉えられている(3)．こういった細胞間の直接的な communication もシナプス機能に重要な役割を果たしていることがわかってきたのである．

Eph 受容体はチロシンキナーゼ受容体の中でも最大のファミリーを構成しており，膜結合性リガンドである ephrin と結合する．この結合は二方向性シグナル伝達を引き起こすので，受容体側，リガンド側の両細胞にシグナルが伝達することになる．2003 年には，シナプス周囲アストロサイトの突起にある ephrin-A3 が海馬錐体細胞の EphA4 受容体に結合し spine の形態を調節することが見いだされた(4)．Ephrin/Eph 受容体シグナルはこれまでも，細胞遊走やパターン化，軸索ガイダンスなど，中枢神経系発達における機能が注目されていたが，この報告はシナプス機能調節における neuron-astrocyte の直接的な cross talk について分子機構まで踏み込んだ重要な報告となった．

Neuron-astrocyte communication における ephrin-A3/EphA4 受容体シグナルの分子機構についてはさらに研究が進み，EphA4 活性化が $\beta$1-integrin の下流シグナルを阻害し spine を安定化すること(5)，アストロサイトでは ephrin-A3 を介して L-Glu トランスポーターの発現が抑制されることも見いだされた(6)．

これらの知見に基づき 2009 年には，neuron-astrocyte communication における ephrin-A3/EphA4 受容体シグナルがシナプス可塑性の制御にも関わっていることが明らかとなった．すなわち，ephrin-A3/EphA4 受容体シグナルが astrocyte の L-Glu トランスポーターの発現を制御することにより，海馬 CA1-CA3 シナプスにおける LTP を調節していることがわかったのである(7)．Ephrin-A3 ノックアウトマウスでは海馬を介した学習が阻害されることも確認されている(6)．

このような neuron-glia communication を介したシナプス機能調節は astrocyte－neuron 間にとどまるものであろうか？　大脳皮質細胞の初代培養を行うと，シナプスへのミクログリアの集積がしばしば観察される．ミクログリアの病態生理学的な重要性が注目されているが，脳内環境変化に感受性の高いミクログリアもアストロサイト同様，シナプス機能調節に関連しているかもしれない．

## 文　献

1) Araque A, et al. Trends Neurosci. 1999;22:208-215.
2) Haydon PG. Nat Rev Neurosci. 2001;2:185-193.
3) Nishida H, et al. J Neurosci. 2007;27:331-340.
4) Murai KK, et al. Nat Neurosci. 2003;6:153-160.
5) Bourgin C, et al. J Cell Biol. 2007;178:1295-1307.
6) Carmona MA, et al. Proc Natl Acad Sci U S A. 2009;106:12524-12529.
7) Filosa A, et al. Nat Neurosci. 2009;12:1285-1292.

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# Deficiency of the *Erc/mesothelin* gene ameliorates renal carcinogenesis in *Tsc2* knockout mice

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**Genetic crossing experiments were performed between tuberous sclerosis-2 (*Tsc2*) KO and expressed in renal carcinoma (*Erc*) KO mice to analyze the function of the *Erc/mesothelin* gene in renal carcinogenesis. We found the number and size of renal tumors were significantly less in *Tsc2*+/−;*Erc*−/− mice than in *Tsc2*+/−; *Erc*+/+ and *Tsc2*+/−;*Erc*+/− mice. Tumors from *Tsc2*+/−;*Erc*−/− mice exhibited reduced cell proliferation and increased apoptosis, as determined by proliferating cell nuclear antigen (Ki67) and TUNEL analysis, respectively. Adhesion to collagen-coated plates *in vitro* was enhanced in *Erc*-restored cells and decreased in *Erc*-suppressed cells with siRNA. Tumor formation by *Tsc2*-deficient cells in nude mice was remarkably suppressed by stable knockdown of *Erc* with shRNA. Western blot analysis showed that the phosphorylation of focal adhesion kinase, Akt and signal transducer and activator of transcription protein 3 were weaker in *Erc*-deficient/suppressed cells compared with *Erc*-expressed cells. These results indicate that deficiency of the *Erc/mesothelin* gene ameliorates renal carcinogenesis in *Tsc2* KO mice and inhibits the phosphorylation of several kinases of cell adhesion mechanism. This suggests that Erc/mesothelin may have an important role in the promotion and/or maintenance of carcinogenesis by influencing cell-substrate adhesion via the integrin-related signal pathway. (*Cancer Sci*, doi: 10.1111/j.1349-7006.2011.01846.x, 2011)**

Expressed in renal carcinoma (*Erc*) was identified as an inducible gene during renal carcinogenesis in the Eker (tuberous sclerosis-2 [*Tsc2*] mutant) rat. The background of this research originates from our studies of the mechanism of multistep carcinogenesis in an animal model involving the *Tsc2* mutant gene using the Eker rat.[1–4] Development of hereditary renal carcinomas in the Eker rat is initiated by a somatic second hit[5] of the *Tsc2* gene. To elucidate the "steps" involved in *Tsc2*-deficiency, genes induced during renal carcinogenesis were cloned in the Eker rat and *Erc* was identified as a novel gene. Subsequently, it was revealed that *Erc* is a homologue of the human *mesothelin* gene.[6] Also, Erc protein is a homologue of a 31-kDa megakaryocyte potentiating factor (MPF), which can stimulate the megakaryocyte colony-forming activity of murine interleukin-3 in mouse bone marrow cell culture. Moreover, Erc protein was also cloned as an antigenic mesothelin for the monoclonal antibody K1 raised against ovarian cancer.[7–9]

Erc/mesothelin protein is a glycosyl phosphatidylinositol (GPI)-anchored membrane glycoprotein that is expressed in normal mesothelial cells. It is also highly expressed in several species of malignant tumors, such as mesothelioma as well as ovarian and pancreatic cancers.[10–13] Its primary product, a 71-kDa precursor of protein, can be physiologically cleaved by a furin-like protease into two fragments. A 31-kDa amino-terminal fragment (MPF, described hereafter as N-Erc/mesothelin) is released into the extra-cellular fluids, while a 40-kDa carboxy-terminal fragment (C-Erc/mesothelin) remains in the cell membranes.[10–16] Soluble N-Erc/mesothelin in serum is already being utilized as a diagnostic tumor marker[14–16] and anti-C-Erc/mesothelin immunotoxin therapy has been reported to be effective for mesothelioma and some C-Erc/mesothelin-expressing cancers.[10] Several *in vitro* studies have suggested that activation of cancer-associated signaling pathways increases Erc/mesothelin expression, and Erc/mesothelin may play a role in tumor adhesion, dissemination, metastasis and resistance against cell death.[9,10,17–22] However, mutant mice in which both copies of the mesothelin gene were inactivated showed no detectable abnormalities when compared with wild-type littermates.[23] Thus, it is conceivable that Erc/mesothelin may have a specific role in carcinogenesis as well as pathogenesis.

The *Erc/mesothelin* gene, described hereafter as *Erc*, is also highly expressed in renal tumor cells from *Tsc2* KO heterozygous mutant (*Tsc2*+/−) mice, that develop hereditary renal tumors presenting as cysts, cyst-adenomas, and solid adenomas that histologically resemble those in the Eker rat.[24]

In this study, *Tsc2*+/−;*Erc*−/− double mutant mice were generated through meiotic recombination and several renal tumor cell lines were established. The phenotypes of the *Tsc2* KO mice with or without *Erc* expression were compared and functions of the *Erc* gene in carcinogenesis were analyzed *in vivo* and *in vitro*. We report here that the development of renal tumors was significantly reduced in *Tsc2*+/−;*Erc*−/− mice, as compared to *Tsc2*+/− (*Erc* WT) or *Tsc2*+/−;*Erc*+/− mice and the several phosphorylation events mediated by integrin and the mammalian target of rapamycin (mTOR) were disrupted in *Tsc2*;*Erc* double deficient renal tumor cells.

## Materials and Methods

**Gene targeting and generation of *Erc* knockout mouse and crossing with *Tsc2* knockout mouse.** Genomic DNA clones covering *Erc* were prepared from a mouse genomic DNA library and used for construction (see Data S1 and Fig. S1 for details).

**Tumor measurement and tissue preparation.** Mice were sacrificed at 18 months of age. The visible tumors on the renal surface were counted and measured with a caliper for length and width. The size of a tumor was defined to be the tumor's mean diameter: (length [mm] + width [mm])/2. Mice were divided into three groups according to the size of their largest tumor: small (<3 mm), large (3–10 mm) and extra-large (>10 mm). Tissues were fixed in 10% neutral formalin and paraffin sections (3 μm each) were prepared for examination.

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**Cell adhesion assay.** The assay was performed according to a method described previously[25] with minor modifications and with type I collagen-coated 24-well plates (Iwaki, Tokyo, Japan). Briefly, after blocking nonspecific adhesion with 1% bovine serum albumin in PBS, $1 \times 10^5$ cells suspended in 1.0 mL of 10% FCS/RPMI-1640 were added to each well and the cells were allowed to adhere for 1 h at 37°C in a 5% $CO_2$ incubator. After washing with PBS, the remaining cells were stained with 0.5% crystal violet in 20% methanol for 30 min and then washed away with water. The stained cells were solubilized in 20% acetic acid and the absorbance of the solution was read in a microplate spectrophotometer (Benchmark Plus; Bio-Rad, CA, USA) at 595 nm. Three independent experiments were performed in quadruplicate.

**Transplantation assay.** BALB/c nude mice were injected subcutaneously with $5 \times 10^6$ tumor cells in 100 μL of serum-free medium. After tumors appeared, the tumors were measured weekly with a caliper for length, width and height and the volume was calculated using the following formula: tumor volume ($mm^3$) = length (mm) × width (mm) × height (mm)/2.

**Statistical analysis.** All discrete values, expressed as mean ± SEM, were analyzed using Student's *t*-test. *P*-values of <0.05 were considered statistically significant.

See Supporting Information (Data S1) for additional methods.

## Results

**Establishment of *Tsc2;Erc* double knockout mice.** *Trans*-compound double heterozygous mutant (*Tsc2*+/− and *Erc*+/−) male mice were mated with C57BL/6J (WT) females (Fig. 1a). Of the 109 offspring, there was a single mouse carrying both *Tsc2* and *Erc* mutations that is a *cis*-compound double heterozygous mutant (Fig. 1b, white star), provisionally designated Erc109.

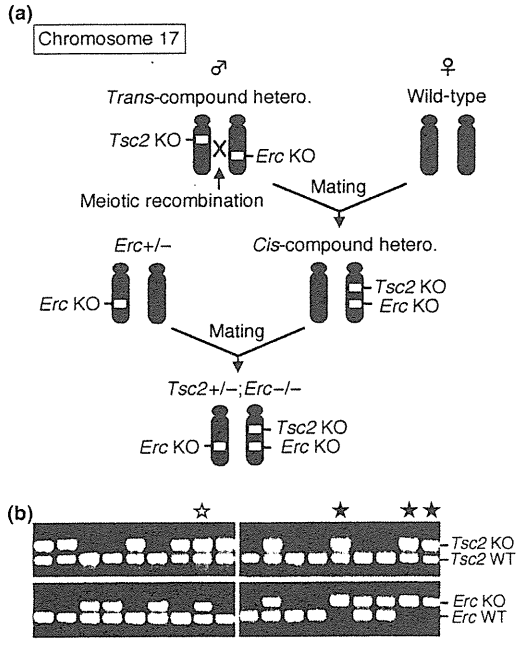


**Fig. 1.** Generation of tuberous sclerosis-2 (*Tsc2*); expressed in renal carcinoma (*Erc*) double KO mouse. (a) Outline of intercrosses. Only *Erc*-deficient mouse is denoted after second mating. (b) Genotyping of genomic DNA by PCR. Representative results of offspring from first cross (left panels) and second cross (right panels) are shown. Upper and lower panels show genotypes of *Tsc2* and *Erc*, white and black stars indicate the *Tsc2*+/−;*Erc*+/− (*cis*-compound heterozygous) mutant and the *Tsc2*+/−;*Erc*−/− mutant, respectively.

*Tsc2*+/−;*Erc*−/− mice (Fig. 1b, black stars) were established by mating Erc109 (*Tsc2*+/−;*Erc*+/−) with *Erc* KO mice (Fig. 1a). Absence of Erc protein in *Tsc2*+/−;*Erc*−/− mice was confirmed immunohistochemical staining of the lung mesothelium (Fig. 2a) using anti-mouse C-Erc/mesothelin rabbit polyclonal antibody (Fig. S2).

**Reduced renal tumor development in *Tsc2*+/−;*Erc*−/− mice.** Renal tumor development was examined in *Tsc2*+/− (*Erc* WT), *Tsc2*+/−;*Erc*+/− and *Tsc2*+/−;*Erc*−/− mice (Fig. 2b–f). Renal tumors were developed in all of the above mice at 18 months of age (Fig. 2b,c,f). However the number (Fig. 2d) and size (mean diameter; Fig. 2b,e,f) of visible tumors on the renal surface were significantly reduced in the *Tsc2*+/−;*Erc*−/− mice. In *Tsc2*+/− as well as *Tsc2*+/−;*Erc*+/− mice, frequent development of tumors of large-size (≥3 mm) were observed in both females and males. There were 59.3% (16 of 27) female and 58.3% (14 of 24) male *Tsc2*+/− mice and 58.6% (17 of 29) female and 57.1% (16 of 28) male *Tsc2*+/−;*Erc*+/− mice that developed large-size tumors. In contrast, only two of 20 females (10.0%) and one of 15 males (6.7%) of *Tsc2*+/−;*Erc*−/− mice showed such large-size tumors (Fig. 2f), although they commonly exhibited carcinogenesis. Moreover, extra-large-size (>10 mm) tumors were seen in *Tsc2*+/− and *Tsc2*+/−;*Erc*+/− mice (11 and 13 cases, respectively) but were not found in *Tsc2*+/−;*Erc*−/− mice (Fig. 2b,f). These observations suggest that the progression of renal tumors in *Tsc2* mutant mice was suppressed by *Erc*-deficiency.

**Decreased proliferation in renal tumors from *Tsc2*+/−;*Erc*−/− mice.** To elucidate the cellular basis for the effects of *Erc*-deficient on proliferation and apoptosis of renal tumors, the paraffin-embedded renal tumor sections were stained with anti-mouse Ki67 (a proliferating cell nuclear antigen) by immunohistochemistry and TUNEL analysis, respectively (Fig. 3). Tumors derived from *Tsc2*+/−;*Erc*−/− mice not only were significantly reduced tumor cell proliferation (Fig. 3a) but also showed increased apoptosis (Fig. 3b) compared with tumors from *Tsc2*+/−;*Erc*−/− mice although *Erc*-deficient tumors exhibited a mild increase in apoptosis status (Fig. 3c).

**Positive effects of Erc on collagen-mediated cell-substrate adhesion in renal tumor cell lines.** To conduct an *in vitro* functional analysis of Erc, renal tumor cell lines were established from *Tsc2*+/−;*Erc*−/− mice. Then, *Erc* expression was restored in one of the established cell lines (DE42L-T1-9) by stable transduction of an *Erc* expression vector and the expression of Erc protein was shown by Western blot (Fig. 4a). *Erc*-restored cells (T1-9Ep10 and T1-9Ep13) were found to be more competent to adhere on the collagen-coated plates than *Erc*-deficient (parental and empty-vector) cells (Fig. 4b). To ascertain that the increased adhesion was due to the function of Erc, the expression of Erc in *Erc*-restored cells was re-suppressed by RNAi and the effect of Erc-suppression was verified by RT-PCR and Western blot (Fig. 4c). The adhesion of these cells on collagen-coated plates was significantly reduced by the suppression of *Erc* (Fig. 4d), confirming that Erc positively regulates cell-substrate adhesion.

**Suppression of tumorigenesis with *Erc*-suppression in *Tsc2*-deficient renal tumor cells.** *Erc*-suppressed cells were established by stable expression of shRNA from the MKOC1-277 cell line that is a *Tsc2*-deficient mouse renal tumor cell line with highly expressed *Erc* (Fig. 5a). When *in vivo* tumorigenicity was examined by subcutaneous injections of cells into nude mice, tumors generated from the *Erc*-suppressed cells were smaller and paler than those from the control shRNA cells that showed robust tumorigenesis (Fig. 5b,c). Conversely, when *Erc*-restored cells were assayed, they exhibited more vigorous growth compared with *Erc*-deficient (empty-vector) cells (Fig. S3). These data suggest that Erc exerts a positive effect on tumorigenicity.


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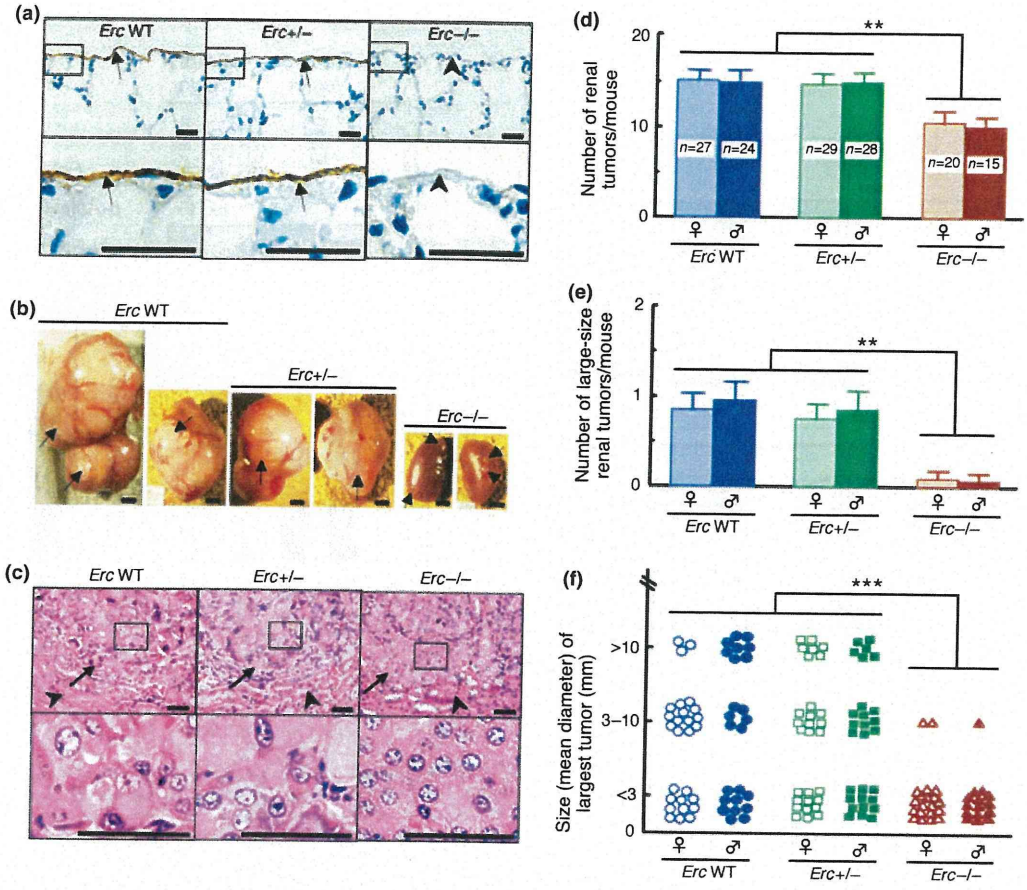


**Fig. 2.** Reduction of the number and size of renal tumors in expressed in renal carcinoma (*Erc*)-deficient mice. (a) Immunohistochemical staining of anti C-Erc/mesothelin show the positive reactions (arrows) in lung mesothelium of tuberous sclerosis-2 (*Tsc2*)+/– (*Erc* WT) and *Tsc2*+/–;*Erc*+/– mice, but not in *Tsc2*+/–;*Erc*–/– mice (arrowheads). Scale bars = 20 μm. (b) Representative macroscopic findings of the renal tumors (arrows) in 18-month-old mice. Scale bars = 3 mm. (c) H&E staining of sections of renal tumors of above mice. Arrows and arrowheads indicate tumors and normal tissues, respectively. Scale bars = 40 μm. (d) The number of renal tumors was evaluated and expressed as average number per mouse. Values are means ± SEM; $^{**}P < 0.01$. Number of mice measured in each group is shown in columns. (e) The number of large-size (≥3 mm) tumors was selected from (d) and expressed as average number per mouse. Values are means ± SEM; $^{**}P < 0.01$. The number of mice measured in each group is the same as (d). (f) The mice were categorized into three groups according to the size (mean diameter) of the largest tumor that the mouse harbored. Points = the largest tumor of each mouse; $^{***}P < 0.001$. The number of mice measured in each group is the same as (d). The large-size and extra-large-size tumor numbers in the *Erc*–/– mice are significantly less when compared with the other mouse strains.

**Modulation of integrin-related signaling by Erc expression.** Integrin β1 is a major subunit of collagen receptors[26] and is required for collagen-mediated proliferation of cancer cells.[27–29] Signals from the integrin complex are transmitted through the phosphorylation of focal adhesion kinase (FAK).[30–32] To determine if Erc expression affects cell adhesion through integrin-related signaling, the expression of integrin β1 and the phosphorylated status of downstream molecules were compared among the indicated cell lines (Fig. 6). As previously reported, two major bands were observed in Western blots of integrin β1,[33–37] namely a partially glycosylated 115 kDa precursor and a fully glycosylated 135 kDa mature form. These bands were disappeared or abolished and a band of core peptide (86 kDa) was appeared upon mild (2 μg/mL) tunicamycin (a *N*-glycosylation inhibitor) treatment, confirming the characteristics of integrin β1 (Fig. S4). Mature integrin β1 was dominant in *Erc*-deficient (parental and empty-vector) cells, while the expression of integrin β1 shifted to the precursor in *Erc*-restored cells (Fig. 6a). Reciprocally, levels of the precursor integrin β1 were decreased in *Erc*-suppressed cells compared with *Erc*-expressed (parental and control shRNA) cells (Fig. 6b). Although direct evidence has not yet been obtained, it is plausible that the expression of mature integrin β1 may be regulated by feedback from cell adhesion machinery regulated by Erc.

The level of phosphorylation of FAK (Tyr925) correlated with Erc expression in cells tested (Fig. 6a), suggesting that the signals downstream of integrin are upregulated by Erc. Further tests were conducted for the phosphorylation of Akt (Ser473), S6K (Thr389) and rpS6 (Ser235/236) with three *Tsc2*-related molecules implicated in insulin signaling and mTOR pathway.[38,39] In *Erc*-restored cells, phosphorylation of Akt (Ser473) and rpS6 (Ser235/236) were found to be more robust while phosphorylation of S6K (Thr389) also was induced although to a lesser extent (Fig. 6a). In other words, the phosphorylation of rpS6 (Ser235/236), catalyzed by S6K as is generally known, was shown to be remarkably weaker than S6K (Thr389) in *Erc*-deficient cells and induced in *Erc*-restored cells. The level of phosphorylation of signal transducer and activator of transcription protein 3 (Stat3; Tyr705) is constitutively higher in *Erc*-restored cells than in *Erc*-deficient cells. Positive effects of Erc on these phosphorylated events were also verified in *Erc*-suppressed cells compared with *Erc*-expressing cells (Fig. 6b).

To investigate the molecular basis for the increase in cell adhesion in the *Erc*-restored cells, the cells were treated with

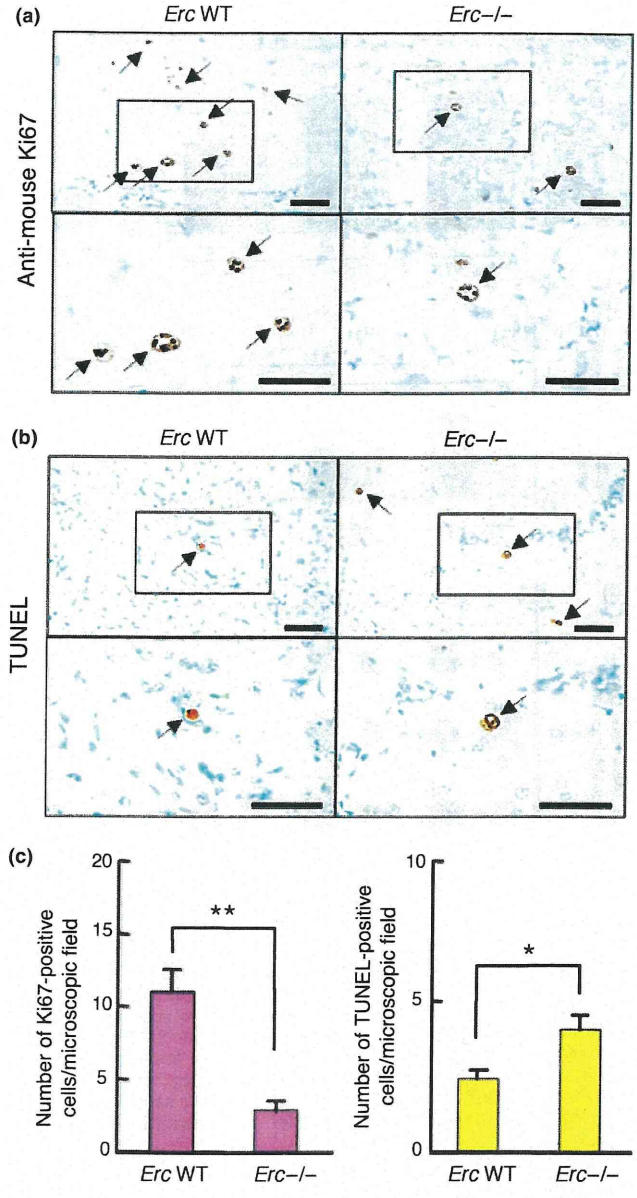


**Fig. 3.** Expressed in renal carcinoma (*Erc*)-deficient decreases tumor growth by impairing cell proliferation. (a) Proliferation was assessed by immunohistochemistry staining on sections of paraffin-embedded renal tumors of the indicated genotype with Ki67 antibody. Positive cells appear brown (arrows). Scale bars = 40 μm. (b) TUNEL-stained sections of paraffin-embedded renal tumors of the indicated genotype. Apoptotic cells appear brown (arrows). Scale bars = 40 μm. (c) The number of Ki67-positive cells (left panel) or TUNEL-positive cells (right panel) per microscopic field was evaluated as described in the Data S1. Values are means ($n$ = 30 images per genotype) ± SEM; $^{**}P < 0.01$; $^{*}P < 0.05$.

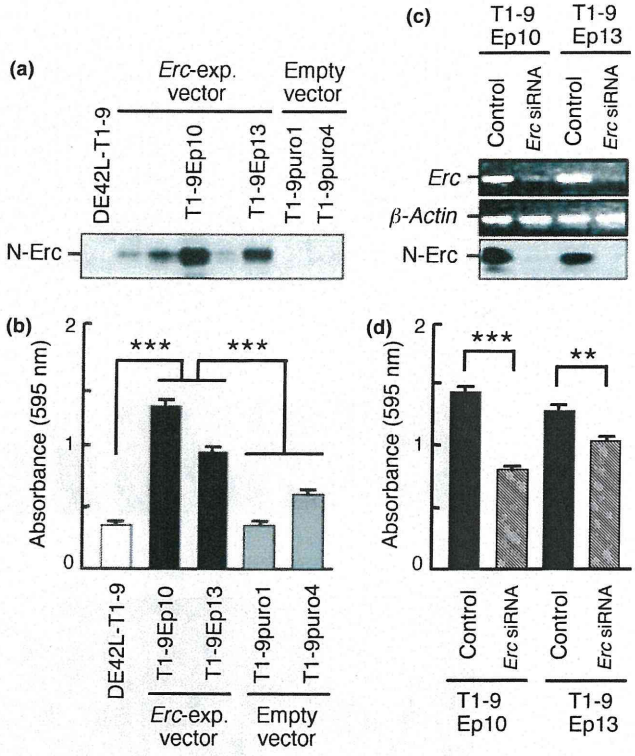


**Fig. 4.** Expressed in renal carcinoma (*Erc*)-expression enhanced cell adhesion to collagen-coated plates. (a) Western blot analysis showed the level of N-Erc expression in the cell lines used for cell adhesion assay. Name of each cell used for further analysis is shown. (b) The adhesion was enhanced in stable *Erc*-restored cells (T1-9Ep10 and T1-9EP13) compared with *Erc*-deficient parental cell line (DE42L-T1-9) and empty-vector cells (T1-9puro1 and T1-9puro4). Values are means ± SEM; $^{***}P < 0.001$. Three independent experiments were performed in quadruplicate. (c) RT-PCR (*Erc* and *β-Actin*) and Western blot analysis (N-Erc) showed the effects of *Erc*-suppression when treated with *Erc* siRNA in *Erc*-restored cells. (d) In contrast to results in (b), the adhesion was decreased in *Erc*-restored cells treated with siRNA. Values are means ± SEM; $^{***}P < 0.001$; $^{**}P < 0.01$. Three independent experiments were performed in quadruplicate.

DMSO (control), 10 μM Akt-I-1/2 (an Akt inhibitor) or 0.1 μM wortmannin (a phosphatidylinositol-3-OH kinase [PI3K] inhibitor), respectively, and then measured the activation of the Akt and the cell adhesion. As shown in Figure 7a, the phosphorylation of Akt (Ser473) was suppressed completely but the phosphorylation of FAK (Tyr925) was not affected in the cells that were treated with an Akt-I-1/2 or wortmannin. The cell adhesion to collagen-coated plates was remarkably decreased in *Erc*-restored cell (T1-9Ep10) treated by both inhibitors compared with control (DMSO) cells (Fig. 7b). These suggest that the activation of Akt by Erc is dependent on PI3K and the cell adhesion positively regulated by PI3K-Akt pathway because phosphorylation of FAK was not affected by Akt-inhibitor or PI3K-inhibitor treatment.

## Discussion

The *Erc* gene is highly expressed in renal tumor cells compared to normal renal cells of *Tsc2*+/− KO mice. Our newly generated *Tsc2*;*Erc* double KO mice made it feasible to investigate the function of Erc in carcinogenesis in a mouse tumor model. The results clearly showed that deficiency of Erc decreased the number and size of renal tumors, and reduced cell proliferation and increased apoptosis in *Tsc2* KO mice, inhibited cell adhesion to collagen-coated plates, and suppressed tumor formation in nude mice. We also showed that Erc influenced the expression of integrin β1 and phosphorylation of several downstream proteins, such as FAK, Akt, rpS6 and Stat3.

The *Tsc2* KO mice and Eker rats, both of which are *Tsc2* heterozygous mutants, develop renal tumors through loss of *Tsc2* in



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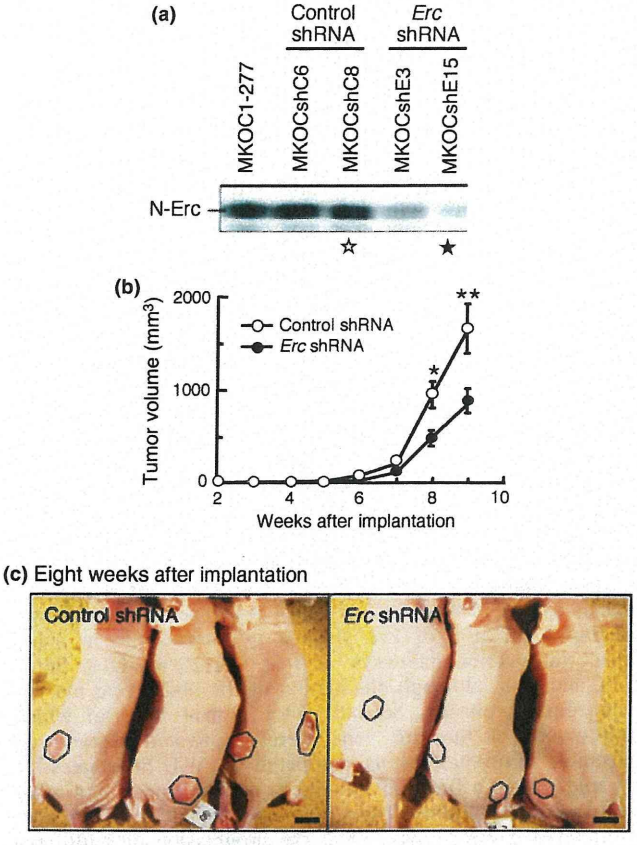


**Fig. 5.** Expressed in renal carcinoma (*Erc*)-deficiency suppressed tumorigenicity of renal tumor cells transplanted in nude mice. (a) Western blot analysis showed the level of N-Erc expression in the stable *Erc*-suppressed cell lines. Black and white stars indicate the cells used for tumorigenicity assay shown below. (b) The tumor volumes were suppressed in stable *Erc*-suppressed (MKOCshE15, black circles) cells compared with control-shRNA (MKOCshC8, white circles) cells after implantation into nude mice. Values are means ± SEM ($n$ = 6); $^{**}P < 0.01$; $^{*}P < 0.05$. (c) Macroscopic appearance of tumors was shown at 8 weeks after implantation. Tumor areas are marked with hexagons. Scale bars = 10 mm.

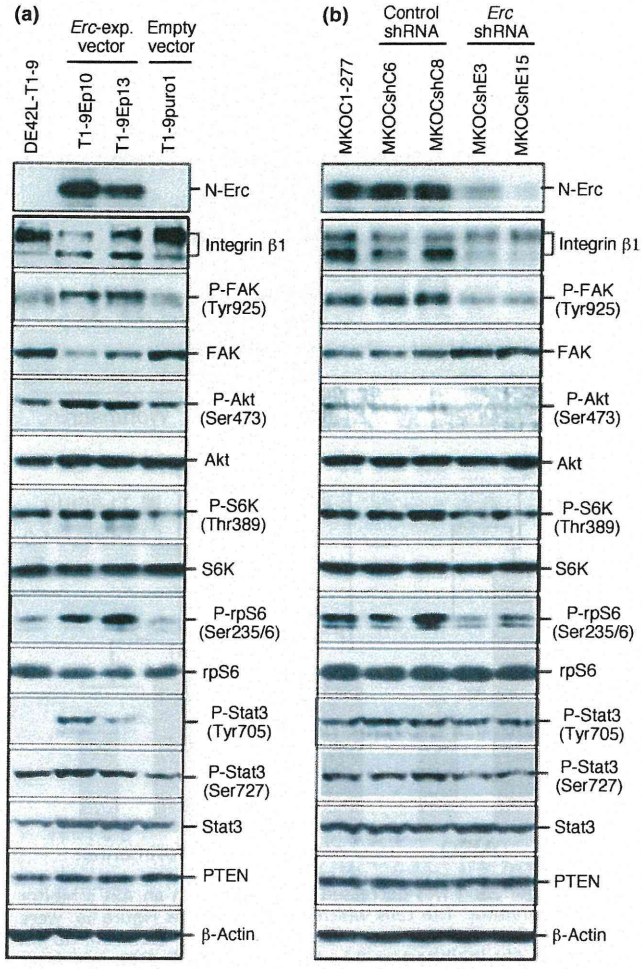


**Fig. 6.** Expressed in renal carcinoma (*Erc*) expression affects integrin-related signal. Western blot analysis was performed with indicated antibodies; the concentrated culture supernatant lysates were used for N-Erc and whole-cell lysates were used for other antibodies. (a) The stable *Erc*-restored cells (central two lanes) increased the precursor integrin β1 (lower-band) and the level of phosphorylation of focal adhesion kinase (FAK), Akt, S6K, rpS6 and Stat3 compared with *Erc*-deficient parental cell line (left lane) and the empty-vector cell (right lane). (b) The stable *Erc*-suppressed cells (right two lanes) decreased the precursor integrin β1 (lower-band) and the level of phosphorylation of FAK, Akt, S6K, rpS6 and Stat3 compared with *Erc*-express (WT) parental cell line (left lane) and the control-shRNA cells (central two lanes).

the WT allele and in multi-steps.[1–5] The human TSC disease is caused by germ-line mutations in either the *TSC1* or *TSC2* gene, with numerous individual tumors generally arising due to somatic ''second-hit'' mutations or loss of heterozygosity[24,40] similar to the above animal models. The resulting tumors in humans and in animal models display elevated mTOR signaling, leading to the enhanced phosphorylation of S6K and rpS6.[1–4,41,42] Erc is highly expressed in renal tumor cells of Eker rats and *Tsc2* KO mice[6,24] but the state of Erc has not been reported in human TSC disease.

The mTOR pathway has a pivotal function in the coordination of cell metabolism, cell growth and cell proliferation[40–44] but another pathway may be involved in *Tsc2* mutant animal models. It has been reported that the administration of rapamycin alone to *Tsc2*-mutant animal models (KO mice and Eker rats) with established tumors results in tumor regression. This however, is characterized with residual tumor or failure to eradicate microscopic pre-tumorous lesions.[45–47] These results suggested the existence of other pathways involved in the *Tsc2*-mutant, in addition to the mTOR axis. The *Tsc2*;*Erc* double KO mice exploited here allowed us to investigate this putative pathway without the confounding effects of possible drug resistance. Our results strongly supported the existence of another pathway, in addition to the mTOR axis.

Recently, it was reported that hotspots of GPI-anchored proteins and integrin nanoclusters were involved in cell adhesion.[48] This was corroborated by our results that showed the expression of Erc affected the pattern of integrin and phosphorylation of several kinases because Erc is one of the GPI-anchored proteins. The new pathway displaying an Erc-cell adhesion mediated tumor-proliferation function may exist, but no association between Erc and integrin-related signal has been reported. Although Erc expression is associated with the decrease in the amount of mature integrin β1, this phenomenon may be caused by some feedback mechanism from activated cell-adhesion machinery partially regulated by Erc.

Signals from the integrin complex are transmitted through the phosphorylation of FAK.[30–32] Akt is a *Tsc2*-related molecule

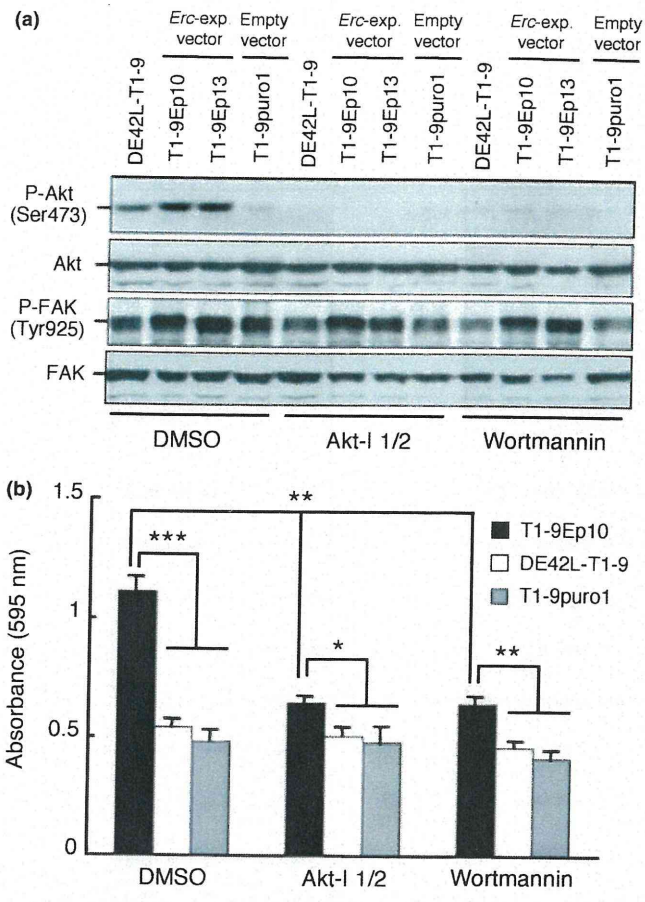


**Fig. 7.** Cell adhesion was decreased by treated with an Akt inhibitor and a PI3K inhibitor. The cells which are the same as Figure 6a, were divided into three groups and treated with DMSO (control), 10 μM Akt-I-1/2 (an Akt inhibitor) or 0.1 μM wortmannin (a PI3K inhibitor), respectively. (a) Western blot analysis was performed with indicated antibodies. The phosphorylation of Akt (Ser473) was suppressed completely with both the inhibitors but the phosphorylation of focal adhesion kinase (FAK; Tyr925) was not affected. (b) The cell adhesion to collagen-coated plates was remarkably decreased in *Erc*-restored cell treated by both inhibitors compared with control (DMSO) cells. Values are means ± SEM; $***P < 0.001$, $*P < 0.01$, $*P < 0.05$. Three independent experiments were performed in quadruplicate.

implicated in insulin signaling[38,39] known to be activated by cell adhesion-related signaling.[49] Our results showed that the level of phosphorylation of FAK (Tyr925), Akt (Ser473) and rpS6 (Ser235/236) were decreased with *Erc*-deficient cells (both *Erc* KO and *Erc* knock down) while the phosphorylation of S6K (Thr389) was reduced to a lesser extent, suggesting that integrin signaling and downstream proteins were upregulated by Erc.

It is well known that *Tsc2*-deficiency activates the mTORC1-S6K pathway as well as the downregulation of Akt.[50] However, our results showed the further downregulation of phosphorylation of Akt (Ser473) by *Erc*-deficiency in *Tsc2*-deficient cells and upregulation in *Erc*-restored cells *in vitro*, suggesting that *Erc*-deficiency is relevant to the suppression of tumor development in *Tsc2+/−* mice. The function of Erc might support the receptor signaling through the cell adhesion signaling. Deficiencies of *Tsc2* and *Erc* might be co-operatively involved in tumorigenesis in KO mice.

The important role of *Erc* in carcinogenesis has attracted a great deal of attention in recent years.[6–22] Several authors reported that overexpression of Erc can accelerate the proliferation and adhesion of cancer cells using cell lines and xenograft models of cancer.[9,10,17–22] Our previous study showed that *Erc* gene expression silenced by siRNA suppresses tumor growth in the *Tsc2* mutant renal carcinoma model.[51] The activation of specific signaling pathways that are important in cancer can lead to an increase in Erc expression. Binding of ovarian cancer antigen CA125/MUC16 to Erc mediates cell adhesion.[17] The overexpression of Erc in pancreatic cancer cells leads to constitutive activation of the transcription factor Stat3, which results in enhanced expression of cyclin E and cyclin E/cyclin-dependent kinase 2 complex formation, as well as increased G1-S transition.[20] Erc inhibits paclitaxel-induced apoptosis through the PI3K pathway.[21] Erc is also differentially regulated by members of the Wnt signal transduction pathway.[22] Combining these reports with our data, multiple possibilities can be considered. First, the pathological high-expression of Erc in specific malignant tumors of humans and in animal models is considered to be the result of carcinogenesis by the mutant *Tsc2* or other key gene. The pathological high-expression of Erc plays a prominent role in many signal transduction pathways such as CA125/MUC16, Stat3, PI3K and Wnt, although elucidation of the underlying mechanism remains elusive. Second, since mutant mice in which both copies of the Erc gene were inactivated showed no detectable abnormalities as compared to WT littermates,[23] Erc might have a function specific to carcinogenesis and other pathological conditions. Third, the downregulation of an integrin-related pathway may affect the progression of multi-step carcinogenesis and inhibit the development of large tumors in *Tsc2+/−;Erc−/−* mice.

In conclusion, we report here that deficiency of Erc affected the integrin-related signal pathway and suppressed the growth of renal tumors in *Tsc2* KO mice. An understanding of the signaling pathways and mechanisms of Erc-induced tumor cell adhesion, proliferation and survival may elucidate not only the pathogenesis of renal tumors in Tsc2+/− mice, but also the pathogenesis of other malignant tumors in both animal models and humans. Our experimental system of *Tsc;Erc* KO mice and cells is useful to unravel the important role of Erc during carcinogenesis, and further analysis of the Erc pathway may help to develop novel anti-cancer therapies.

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## References


1 Eker R, Mossige J. A dominant gene for renal adenomas in the rat. *Nature* 1961; **189**: 858–9.
2 Hino O, Kobayashi T, Momose S, Kikuchi Y, Adachi H, Okimoto K. Renal carcinogenesis: genotype, phenotype and dramatype. *Cancer Sci* 2003; **94**: 142–7.
3 Hino O, Kobayashi T, Tsuchiya H *et al.* The predisposing gene of the Eker rat inherited cancer syndrome is tightly linked to the tuberous sclerosis (TSC2) gene. *Biochem Biophys Res Commun* 1994; **203**: 1302–8.
4 Kobayashi T, Hirayama Y, Kobayashi E, Kubo Y, Hino O. A germline insertion in the tuberous sclerosis (*Tsc2*) gene gives rise to the Eker rat model of dominantly inherited cancer. *Nat Genet* 1995; **9**: 70–4.
5 Knudson AG. Mutation and cancer: statistical study of retinoblastoma. *Proc Natl Acad Sci U S A* 1971; **68**: 820–3.
6 Hino O, Kobayashi E, Nishizawa M *et al.* Renal carcinogenesis in the Eker rat. *J Cancer Res Clin Oncol* 1995; **121**: 602–5.
7 Kojima T, Oh-eda M, Hattori K *et al.* Molecular cloning and expression of megakaryocyte potentiating factor cDNA. *J Biol Chem* 1995; **270**: 21984–90.
8 Chang K, Pastan I. Molecular cloning of mesothelin, a differentiation antigen present on mesothelium, mesotheliomas, and ovarian cancers. *Proc Natl Acad Sci U S A* 1996; **93**: 136–40.
9 Yamashita Y, Yokoyama M, Kobayashi E, Takai S, Hino O. Mapping and determination of the cDNA sequence of the *Erc* gene preferentially expressed in renal cell carcinoma in the *Tsc2* gene mutant (Eker) rat model. *Biochem Biophys Res Commun* 2000; **275**: 134–40.
10 Hassan R, Bera T, Pastan I. Mesothelin: a new target for immunotherapy. *Clin Cancer Res* 2004; **10**: 3937–42.
11 Scholler N, Fu N, Yang Y *et al.* Soluble member(s) of the mesothelin/megakaryocyte potentiating factor family are detectable in sera from patients with ovarian carcinoma. *Proc Natl Acad Sci U S A* 1999; **96**: 11531–6.
12 Sapede C, Gauvrit A, Barbieux I *et al.* Aberrant splicing and protease involvement in mesothelin release from epithelioid mesothelioma cells. *Cancer Sci* 2008; **99**: 590–4.
13 Li M, Bharadwaj U, Zhang R *et al.* Mesothelin is a malignant factor and therapeutic vaccine target for pancreatic cancer. *Mol Cancer Ther* 2008; **7**: 286–96.
14 Robinson BW, Creaney J, Lake R *et al.* Mesothelin-family proteins and diagnosis of mesothelioma. *Lancet* 2003; **362**: 1612–6.
15 Hino O, Maeda M. Diagnostic tumor marker of asbestos-related mesothelioma. *Environ Health Prev Med* 2008; **13**: 71–4.
16 Shiomi K, Hagiwara Y, Sonoue K *et al.* Sensitive and specific new enzyme-linked immunosorbent assay for N-ERC/mesothelin increases its potential as a useful serum tumor marker for mesothelioma. *Clin Cancer Res* 2008; **14**: 1431–7.
17 Rump A, Morikawa Y, Tanaka M *et al.* Binding of ovarian cancer antigen CA125/MUC16 to mesothelin mediates cell adhesion. *J Biol Chem* 2004; **279**: 9190–8.
18 Hucl T, Brody JR, Gallmeier E, Iacobuzio-Donahue CA, Farrance IK, Kern SE. High cancer-specific expression of mesothelin (MSLN) is attributable to an upstream enhancer containing a transcription enhancer factor dependent MCAT motif. *Cancer Res* 2007; **67**: 9055–65.
19 Que J, Wilm B, Hasegawa H, Wang F, Bader D, Hogan BL. Mesothelium contributes to vascular smooth muscle and mesenchyme during lung development. *Proc Natl Acad Sci U S A* 2008; **105**: 16626–30.
20 Bharadwaj U, Li M, Chen C, Yao Q. Mesothelin-induced pancreatic cancer cell proliferation involves alteration of cyclin E via activation of signal transducer and activator of transcription protein 3. *Mol Cancer Res* 2008; **6**: 1755–65.
21 Chang MC, Chen CA, Hsieh CY *et al.* Mesothelin inhibits paclitaxel-induced apoptosis through the PI3K pathway. *Biochem J* 2009; **424**: 449–58.
22 Prieve MG, Moon RT. Stromelysin-1 and mesothelin are differentially regulated by Wnt-5a and Wnt-1 in C57MG mouse mammary epithelial cells. *BMC Dev Biol* 2003; **3**: 2–11.
23 Bera TK, Pastan I. Mesothelin is not required for normal mouse development or reproduction. *Mol Cell Biol* 2000; **20**: 2902–6.
24 Kobayashi T, Minowa O, Kuno J, Mitani H, Hino O, Noda T. Renal carcinogenesis, hepatic hemangiomatosis, and embryonic lethality caused by a germ-line *Tsc2* mutation in mice. *Cancer Res* 1999; **59**: 1206–11.
25 Gustavsson A, Armulik A, Brakebusch C, Fässler R, Johansson S, Fällman M. Role of the β1-integrin cytoplasmic tail in mediating invasin-promoted internalization of Yersinia. *J Cell Sci* 2002; **115**: 2669–78.
26 Harburger DS, Calderwood DA. Integrin signalling at a glance. *J Cell Sci* 2009; **122**: 159–63.
27 Macias-Perez I, Borza C, Chen X *et al.* Loss of integrin α1 β1 ameliorates Kras-induced lung cancer. *Cancer Res* 2008; **68**: 6127–35.
28 Brenner W, Greber I, Gudejko-Thiel J *et al.* Migration of renal carcinoma cells is dependent on protein kinase Cdelta via beta1 integrin and focal adhesion kinase. *Int J Oncol* 2008; **32**: 1125–31.
29 Park CC, Zhang H, Pallavicini M *et al.* Beta1 integrin inhibitory antibody induces apoptosis of breast cancer cells, inhibits growth, and distinguishes malignant from normal phenotype in three dimensional cultures and in vivo. *Cancer Res* 2006; **66**: 1526–35.
30 Shibue T, Weinberg RA. Integrin β1-focal adhesion kinase signaling directs the proliferation of metastatic cancer cells disseminated in the lungs. *Proc Natl Acad Sci U S A* 2009; **106**: 10290–5.
31 Xia H, Nho RS, Kahm J, Kleidon J, Henke CA. Focal adhesion kinase is upstream of phosphatidylinositol 3-kinase/Akt in regulating fibroblast survival in response to contraction of type I collagen matrices via a beta 1 integrin viability signaling pathway. *J Biol Chem* 2004; **279**: 33024–34.
32 Pylayeva Y, Gillen KM, Gerald W, Beggs HE, Reichardt LF, Giancotti FG. Ras- and PI3K-dependent breast tumorigenesis in mice and humans requires focal adhesion kinase signaling. *J Clin Invest* 2009; **119**: 252–66.
33 Argraves WS, Suzuki S, Arai H, Thompson K, Pierschbacher MD, Ruoslahti E. Amino acid sequence of the human fibronectin receptor. *J Cell Biol* 1987; **105**: 1183–90.
34 Guo HB, Lee I, Kamar M, Akiyama SK, Pierce M. Aberrant *N*-glycosylation of beta1 integrin causes reduced alpha5beta1 integrin clustering and stimulates cell migration. *Cancer Res* 2002; **62**: 6837–45.
35 Bellis SL, Newman E, Friedman EA. Steps in integrin beta1-chain glycosylation mediated by TGFbeta1 signaling through Ras. *J Cell Physiol* 1999; **181**: 33–44.
36 Isaji T, Sato Y, Fukuda T, Gu J. *N*-glycosylation of the I-like domain of beta1 integrin is essential for beta1 integrin expression and biological function: identification of the minimal *N*-glycosylation requirement for alpha5beta1. *J Biol Chem* 2009; **284**: 12207–16.
37 Bellis SL. Variant glycosylation: an underappreciated regulatory mechanism for beta1 integrins. *Biochim Biophys Acta* 2004; **1663**: 52–60. Review.
38 Inoki K, Corradetti MN, Guan KL. Dysregulation of the TSC-mTOR pathway in human disease. *Nat Genet* 2005; **37**: 19–24.
39 Cao Y, Kamioka Y, Yokoi N *et al.* Interaction of FoxO1 and TSC2 induces insulin resistance through activation of the mammalian target of rapamycin/p70 S6K pathway. *J Biol Chem* 2006; **281**: 40242–51.
40 Astrinidis A, Henske EP. Tuberous sclerosis complex: linking growth and energy signaling pathways with human disease. *Oncogene* 2005; **24**: 7475–81.
41 Kobayashi T, Adachi H, Mitani H, Hirayama Y, Hino O. Toward chemotherapy for *Tsc2 mutant* renal tumor. *Proc Jpn Acad* 2003; **79**: 22–5.
42 Shiono M, Kobayashi T, Takahashi R *et al.* The G1556S-type tuberin variant suppresses tumor formation in tuberous sclerosis 2 mutant (Eker) rats despite its deficiency in mTOR inhibition. *Oncogene* 2008; **27**: 6690–7.
43 Guertin DA, Sabatini DM. Defining the role of mTOR in cancer. *Cancer Cell* 2007; **12**: 9–22.
44 Gan B, Yoo Y, Guan J-L. Association of focal adhesion kinase with tuberous sclerosis complex 2 in the regulation of s6 kinase activation and cell growth. *J Biol Chem* 2006; **281**: 37321–9.
45 Kenerson H, Dundon TA, Yeung RS. Effects of rapamycin in the Eker rat model of tuberous sclerosis complex. *Pediatr Res* 2005; **57**: 67–75.
46 Lee L, Sudentas P, Donohue B *et al.* Efficacy of a rapamycin analog (CCI-779) and IFN-gamma in tuberous sclerosis mouse models. *Genes Chromosomes Cancer* 2005; **42**: 213–27.
47 Lee L, Sudentas P, Dabora SL. Combination of a rapamycin analog (CCI-779) and interferon-gamma is more effective than single agents in treating a mouse model of tuberous sclerosis complex. *Genes Chromosomes Cancer* 2006; **45**: 933–44.
48 van Zanten TS, Cambi A, Koopman M *et al.* Hotspots of GPI-anchored proteins and integrin nanoclusters function as nucleation sites for cell adhesion. *Proc Natl Acad Sci U S A* 2009; **106**: 18557–62.
49 Barros CS, Nguyen T, Spencer KS, Nishiyama A, Colognato H, Müller U. β1 integrins are required for normal CNS myelination and promote AKT-dependent myelin outgrowth. *Development* 2009; **136**: 2717–24.
50 Huang J, Wu S, Wu CL, Manning BD. Signaling events downstream of mammalian target of rapamycin complex 2 are attenuated in cells and tumors deficient for the tuberous sclerosis complex tumor suppressors. *Cancer Res* 2009; **69**: 6107–14.
51 Imamura O, Okada H, Takashima Y, Zhang D, Kobayashi T, Hino O. siRNA-mediated Erc gene silencing suppresses tumor growth in Tsc2 mutant renal carcinoma model. *Cancer Lett* 2008; **268**: 278–85.

## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Data S1.** Supporting Methods.

**Fig. S1.** Establishment of expressed in renal carcinoma (*Erc*) KO mice.

**Fig. S2.** Amino acid alignment of the expressed in renal carcinoma (Erc)/mesothelin protein.

**Fig. S3.** Enhanced tumorigenicity of expressed in renal carcinoma (*Erc*)-restored renal tumor cells transplanted in nude mice.

**Fig. S4.** Confirmed the characteristics of integrin β1 by treated with lower concentration of tunicamycin.



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# Feasibility of large-scale screening using N-ERC/mesothelin levels in the blood for the early diagnosis of malignant mesothelioma

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**Abstract.** A large-scale screening involving the measurement of N-ERC/mesothelin levels in blood using an ELISA system for the early diagnosis of malignant mesothelioma (MM) was carried out in individuals with a history of employment at construction sites. Approximately 30,000 subjects were screened. Of the 80 subjects with high-risk values, one male patient was diagnosed as having MM based on a PET study and histopathology. This is the first report of the pre-clinical diagnosis of MM based on blood test screening. In addition, plasma levels of N-ERC/mesothelin may be effectively used for monitoring relapse after surgery.

## Introduction

Mesothelioma is an aggressive tumor arising from the mesothelium, a membrane lining various body cavities, including the pleura, peritoneum and pericardium, and is usually associated with asbestos exposure.

Due to the long incubation period and the short survival time after the onset of asbestos-induced malignant mesothelioma (MM), an early diagnostic system for individuals with a history of asbestos exposure is critically required. Recently, soluble mesothelin-related protein (1) and serum mesothelin have been reported to be potentially useful markers for the early diagnosis of MM (2,3).

A 71-kDa precursor protein of human ERC/mesothelin can be cleaved into a 40-kDa C-terminal fragment as a surface GPI-anchored glycoprotein and a 31-kDa N-terminal fragment as a secreted protein. We focused on this N-ERC/mesothelin, as it is physiologically secreted into the blood, and as a specific ELISA system has been developed for N-ERC/mesothelin (2,3).

In the present study, to develop a pre-clinical diagnostic system for the early detection of MM, the levels of N-ERC/mesothelin, the N-terminal 31-kDa fragment of mesothelin, in blood samples was measured by ELISA as a primary screening method. Chest radiography, chest CT and positron emission tomography (PET) examinations, and also histopathology, were then used as secondary screening examinations for individuals with a history of exposure to asbestos and consequently, a high risk of developing of MM.

## Materials and methods

*Establishment of ELISA for N-ERC/mesothelin and measurement of blood samples.* The sandwich ELISA system used in this report were established as described previously (3). The 7E7 MoAb was used as the capture antibody and the 16K16 MoAb was used as the detecting antibody after conjugation with horseradish peroxidase. Recombinant N-ERC/mesothelin was used as the standard. The protein in the ELISA system was purified from culture supernatants of CHO-K1 cells transfected with ERC/mesothelin cDNA using an anti-ERC/mesothelin PoAb. EDTA plasma was used for this ELISA.

The present study was approved by the Institutional Review Board of Juntendo University School of Medicine, its hospital and Immuno-Biological Laboratories. Written informed consent for participation in the study was obtained from all of the subjects.

## Results

*Case reports of three patients*

*Patient A: clinical history.* The subject was a 71-year-old male who had a history of smoking for 58 years. He did not have any clinical complaints or significant past medical history. The patient had an occupational history of employment as a plumber using asbestos for pipe insulation since the age of 24 years.

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*Key words:* mesothelioma, mesothelin, ELISA, screening

*History of present illness*. The subject underwent a physical examination in June 2007, at which time his plasma N-ERC/mesothelin level was 8.62 ng/ml, which was higher than the cutoff value (3).

He subsequently underwent secondary screening. Although a chest CT revealed bilateral pleural thickening with calcification, no abnormal findings in the abdominal area or interior of the pelvis were observed in an examination performed in October 2007.

In January 2008, a thoracoabdominal CT revealed an anterior mediastinal node and a small amount of pleural effusion, and the amount of ascites was noted to have increased. In May 2008, a PET-CT examination was performed and an accumulation was detected in the anterior mediastinal node and abdominal cavity (Fig. 1). A laparoscopic biopsy was performed in June 2008 and numerous white nodes were detected in the abdominal cavity. A pathological examination confirmed the diagnosis of MM, epithelioid-type (Fig. 2).

During this time period, the patient's blood N-ERC/mesothelin levels increased from 8.62 ng/ml in June 2007 to 13.59 ng/ml in October 2007 and 31.49 ng/ml in April 2008.

*Patient B: clinical history*. The subject was a 68-year-old male. He had suffered from glaucoma since the age of 42, gout since the age of 50 and high blood pressure and hyperlipidemia since the age of 55 years.He had an occupational history of employment as a quality control supervisor in charge of electric machinery that was stored in heat-insulating material (asbestos) in his 20s, in an electrical construction company where spraying with asbestos was carried out in his immediate environment in his 30s, and in a building custodial service using asbestos for caulking projects around pumps from the age of 40.

*History of present illness*. The subject underwent a physical examination each year. In 2007, an abnormality was detected on a plain chest roentgenogram. In November 2008, an abnormality was again perceived on a plain chest roentgenogram. In December 2008, a pleural effusion was detected at a local hospital. In February 2009, thoracentesis was performed, and a cytological examination of the pleural fluid revealed class V cytology.

The patient subsequently visited the clinic of Juntendo University Hospital in the same month, at which time his plasma N-ERC/mesothelin level was 15.6 ng/ml, which was in excess of the cutoff value (3). In April 2009, a pleural biopsy was performed. Histopathological examination confirmed a diagnosis of MM, epithelioid-type. The TNM class was evaluated as pT1bN0M0 and the clinical stage as stage IB. In June, the patient was admitted to the hospital. Surgical removal of the pericardium and diaphragm and left extrapleural pneumonectomy were performed immediately. On day 3 after the surgery, the thoracostomy tube was removed. On day 11, as he was making satisfactory progress, he was discharged from the hospital, at which time his plasma N-ERC/mesothelin level was 1.63 ng/ml, lower than the cutoff value. After discharge from the hospital, he visited the clinic again in June, July, August and December 2009, and February 2010, and no evidence of recurrence was detected. In December 2009, the plasma N-ERC/mesothelin level was 2.64 ng/ml, which was within the normal range.

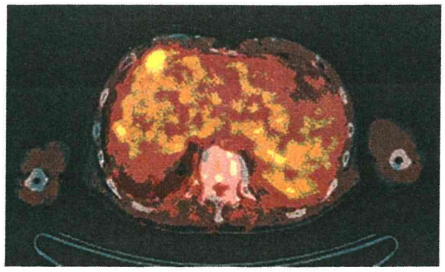

Figure 1. FDG PET/CT scan shows an 18x12-mm nodule located between the diaphragm and liver. It displayed strong FDG tracer uptake.

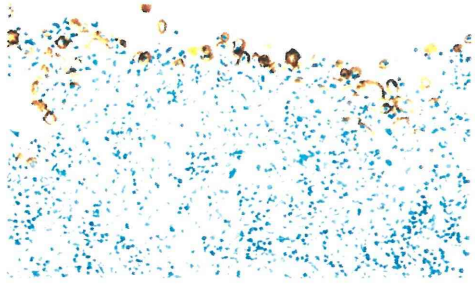

Figure 2. Tumor biopsy from the peritoneum shows mesothelioma, epithelioid-type. Mesothelioma cells exhibited ERC/mesothelin expression on the outer surface.

*Patient C: clinical history*. The subject was a 68-year-old female. She had a previous history of undergoing surgical removal of a chocolate cyst in the right ovary. She had an occupational history of employment as a teacher in a school building with asbestos insulation.

*History of present illness*. The patient visited a local hospital with the chief complaint of dyspnea on exertion and was diagnosed as having a bloody pleural effusion on the left side.

A tumor mass on the chest wall was detected by chest CT. A lung biopsy was performed. Cytological examination of the pleural fluid revealed class III cytology, and biochemical examination revealed a markedly elevated level of hyaluronic acid (646,000 ng/ml). Cytological examination of the lacteal gland discharge revealed class V cytology. In April 2009, the patient visited the clinic of Juntendo University Hospital. A pleural biopsy was performed. Histopathological examination confirmed the diagnosis of malignant pleural mesothelioma. The TNM classification was evaluated as pT4N2M0 and the clinical stage as IV. She was discharged from the hospital, but was re-admitted in May of the same year because of re-accumulation of the pleural fluid, which was treated by drainage. A pleurosclerosis was performed using OK-432 and 5KE. She was discharged from the hospital, but was again admitted in June 2009, at which time her plasma N-ERC/mesothelin level reached 117.50 ng/ml, markedly higher than the cutoff value. A left extrapleural pneumonectomy was performed. On day 3 after the surgery, the thoracostomy tube was removed. On day 9, as she was making satisfactory progress, she was discharged from the hospital. After the surgery, her plasma N-ERC/mesothelin levels were 14.46 ng/ml in December 2009, 7.13 ng/ml

in February 2010 and 8.36 ng/ml in April 2010. She showed no evidence of recurrence until February 2010. In July 2010, she was found to have a tumor recurrence on the chest wall and a metastasis in the contralateral lung, and was re-admitted to the hospital for chemotherapy, at which time her plasma N-ERC/mesothelin level had again increased to 36.03 ng/ml.

*Relationship between the plasma level of N-ERC/mesothelin and the pathogenesis of MM.* Patient A was detected during a large-scale screening of approximately 30,000 subjects affiliated with the Tokyo Doken National Health Insurance Association who had worked at construction sites, and who had undergone a physical examination and submitted blood samples for laboratory analysis. The N-ERC/mesothelin level in each blood sample was measured using our novel ELISA method.

Approximately 80 subjects were identified as belonging to a high-risk group based on the previously reported cutoff value for N-ERC/mesothelin (3). Of these subjects, 30 individuals underwent secondary screening, and one man (patient A) was diagnosed as having MM based on the results of a PET study and histopathology. It is noteworthy that, in this case, although no abnormal findings were observed on a thoracoabdominal CT performed in October 2007, the plasma N-ERC/mesothelin level was already elevated at this time. Thus, this is the first report of the pre-clinical diagnosis of MM based on a blood test.

The two additional cases reported here were not detected to have MM in the large-scale screening study, but they represent appropriate examples to demonstrate the effectiveness of N-ERC/mesothelin as a blood marker for the diagnosis of MM. Patient B was a 68-year-old male who was detected to have an abnormality on a plain chest X-ray during a medical check-up. A histopathological examination confirmed the diagnosis of MM, epithelioid-type. Surgical removal of the pericardium and diaphragm and a left extrapleural pneumonectomy were performed. Before the surgery, the plasma N-ERC/mesothelin level was 15.6 ng/ml; however, following the surgery it decreased to 1.63 ng/ml. There has been no evidence of recurrence since.

Patient C was a 68-year-old female working as a teacher in a school building with asbestos insulation. She had a previous history of surgical removal of a chocolate cyst in the right ovary. She visited our hospital because of dyspnea on exertion, and was diagnosed as having a bloody pleural effusion on the left side. Histopathologic examination of a pleural biopsy confirmed the diagnosis of malignant pleural mesothelioma. A left extrapleural pneumonectomy was performed. Before the surgery, the plasma N-ERC/mesothelin level was 117.50 ng/ml, while after the surgery it decreased to 7.13 ng/ml. However, 11 months later, the patient developed a tumor recurrence on the chest wall and a metastasis in the contralateral lung, at which time her plasma N-ERC/mesothelin level had again increased to 36.03 ng/ml.

As described above, the plasma level of N-ERC/mesothelin may be a useful blood marker for the early diagnosis of MM, and may be particularly effective for monitoring patients for relapse after surgery.

Early diagnosis of MM may become possible by measurement of the blood N-ERC/mesothelin level as a routine laboratory test in high-risk populations with a history of exposure to asbestos.

## Discussion

This study showed the effectiveness of N-ERC/mesothelin as a blood marker for MM. At present, two lines of clinical studies for mesothelin-bearing tumors are ongoing: a phase I trial of the anti-mesothelin immunotoxin SS1P in patients with mesothelioma, ovarian and pancreatic cancer (4), and a phase I clinical trial of MORAb-009, which is a chimeric antibody targeting tumor-associated mesothelin (5,6). Additionally, Inami *et al* reported the antitumor activity of the anti-C-ERC/mesothelin monoclonal antibody *in vivo* (7), and Yoshida *et al* reported on the use of PET imaging of 64Cu-labeled Fab for detecting ERC/mesothelin in a mesothelioma mouse model (8).

It would be most useful to combine immunotherapy using an anti-mesothelin antibody directed against mesothelin-bearing tumors and a blood test for ERC/mesothelin.

The screening protocol reported herein is very promising for the early diagnosis of MM in subjects with a history of exposure to asbestos. In addition, measurement of the plasma level of N-ERC/mesothelin may also be useful in the follow-up of patients after surgery for MM.

## Acknowledgements

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## References

1. Robinson BWS, Creaney J, Lake R, Nowak A, Musk AW and De Klerk N: Mesothelin-family proteins and diagnosis of mesothelioma. Lancet 362: 1612-1616, 2003.
2. Shiomi K, Miyamoto H, Segawa T, Hagiwara Y, Ota A and Hino O: Novel ELISA system for detection of NERC/mesothelin in the sera of mesothelioma patients. Cancer Sci 97: 928-932, 2006.
3. Shiomi K, Hagiwara Y, Sonoue K, *et al*: Sensitive and specific new enzyme-linked immunosorbent assay for N-ERC/mesothelin increases its potential as a useful serum tumor marker for mesothelioma. Clin Cancer Res 14: 1431-1437, 2008.
4. Hassan R, Bullock S, Premkumar A, Kreitman RJ, Kindler H, Willingham M and Pastan I: Phase I study of SS1P, a recombinant anti-mesothelin immunotoxin for targeted therapy of mesothelin-expressing mesotheliomas, ovarian and pancreatic cancer. J Clin Oncol 25: 3553, 2007.
5. Hassan R, Ebel W, Routhier E, *et al*: Preclinical evaluation of MORAb-009, a chimeric antibody targeting tumor-associated mesothelin. Cancer Immun 7: 20-29, 2007.
6. Hassan R and Ho M: Mesothelin-targeted cancer immunotherapy. Eur J Cancer 44: 46-53, 2008.
7. Inami K, Abe M, Takeda K, Hagiwara Y, Maeda M, Segawa T, Suyama M, Watanabe S and Hino O: Antitumor activity of anti-C-ERC/mesothelin monoclonal antibody in vivo. Cancer Sci 101: 969-974, 2010.
8. Yoshida C, Sogawa C, Tsuji A, Sudo H, Sugyo A, Uehara T, Hino O, Yoshii Y, Fujibayashi Y, Fukumura T, Koizumi M, Arano Y and Saga T: Development of positron emission tomography imaging by 64Cu-labeled Fab for detecting ERC/mesothelin in mesothelioma mouse model. Nucl Med Commun 31: 380-388, 2010.

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# Impact of Renal Failure on the Tumor Markers of Mesothelioma, N-ERC/mesothelin and Osteopontin

KAZU SHIOMI[1], SATOKO SHIOMI[2], YUJI ISHINAGA[2], MOTOKI SAKURABA[1], YOSHIAKI HAGIWARA[3], KAZUYA MIYASHITA[3], MASAHIRO MAEDA[3], KENJI SUZUKI[1], KAZUHISA TAKAHASHI[4] and OKIO HINO[5]

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**Abstract.**

Individuals at high risk for mesothelioma due to severe exposure to asbestos can be readily identified, and diagnosis of malignant mesothelioma (MM) at an early stage improves its otherwise dismal prognosis (1). However, most patients are diagnosed at a clinically advanced stage because it is difficult to confirm the existence of these tumours with current imaging technology alone. Therefore, there is a strong need to study the characteristics of tumour markers for MM for them to be used more effectively.

We previously studied promising tumour markers for MM, N-ERC/mesothelin (N-ERC) and osteopontin (OPN) (2-11). In the course of these studies, we found that the blood concentration of N-ERC increases with age and that the serum level of N-ERC is elevated in hemodialysis patients. These findings suggested renal function influences the concentrations of these tumour markers. Furthermore, most patients with MM are elderly and have various degrees of renal dysfunction, and their renal function sometimes becomes worse during chemotherapy. Therefore, it is important to determine the relations between the concentrations of tumour markers and the extent of renal failure to make an accurate early diagnosis and to judge the effect of chemotherapy.

In this study, we have examined whether and to what extent renal dysfunction influences the blood concentrations of N-ERC and OPN, tumour markers of MM.

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*Key Words:*

## Materials and Methods

*Study samples and assays.* This study was conducted as a part of our research program to develop tumour markers for mesothelioma (2, 3). This program was approved by the Institutional Review Board of Juntendo University School of Medicine, its hospital, Shouwakai Hospital, National Organization Tokyo Hospital, Hyogo Prefectural Amagasaki Hospital, Japan Anti-Tuberculosis Association Fukujuji Hospital, Yokosuka Kyosai Hospital, St. Marianna University School of Medicine, Tohoku University Hospital, and Immuno-Biological Laboratories, Ltd. This study primarily uses data from patients in the first two hospitals listed above.

Blood sample was collected once from patients with various levels of renal dysfunction, including those on dialysis, and healthy volunteers when they first visited hospital right after their informed consent were given, period from August 2005 till the end of March 2008.

We used the new estimated glomerular filtration rate (eGFR) for Japanese and the chronic kidney disease (CKD) classification, which define renal function more specifically and clearly, as follows: eGFR (ml/min/1.73 $m^2$)=194×$Cr^{-1.094}$×$age^{-0.287}$ (female: ×0.739), with the CKD classifications according to eGFR in ml/min/1.73 $m^2$ of stage I ≥90, stage II 60-89, stage III 30-59, stage IV 15-29, and stage V <15.

The serum levels of N-ERC and the plasma levels of OPN were measured by a specialist at the Juntendo University School of Medicine with an N-ERC/mesothelin ELISA Kit (7-16) (Immuno-Biological Laboratories, Ltd., Gunma, Japan) and a Human Osteopontin Assay Kit (Immuno-Biological Laboratories), respectively.

Table I. *Characteristics of patients and volunteers.*

| | Renal dysfunction (n=32) | Hemo-dialysis (n=22) | Healthy volunteers (n=102) |
|---|---|---|---|
| Age (years) | 38-83 (67.2±11)* | 41-86 (64.4±12) | 30-79 (52.9±14) |
| Gender | | | |
| Male | 23 | 12 | 52 |
| Female | 9 | 10 | 50 |
| Comorbidity | | | |
| DM | 17 | 7 | – |
| HT | 21 | 16 | – |
| Other | 3 | 4 | – |
| Stage | | | |
| I | 4 | | |
| II | 7 | | |
| III | 12 | | |
| IV | 5 | | |
| V | 4 | | |

*Values are means±SD. DM, Diabetes mellitus; HT, hypertension.

*Statistical analysis.* ELISA data were analysed with JMP and SAS version 8.1.3 (SAS Institute, Cary, NC) and Graph Pad Prism 4.0 (GraphPad Software, San Diego, CA). To compare serum and plasma levels between groups, the Mann-Whitney test was used. Linear regression analysis was used to analyse the trend of increasing serum and plasma levels with increasing CKD stage. Furthermore, multiple linear regression analysis was used to estimate the important variables that caused the differences in concentrations of these markers between the CKD group and volunteers. A value of $p<0.05$ was considered statistically significant.

## Results

Blood samples were obtained from 32 patients with chronic renal dysfunction, 22 of whom were on hemodialysis (CKD group), and from 102 healthy volunteers. Characteristics of the patients are shown in Table I. Serum concentrations of N-ERC and plasma concentrations of OPN in the CKD group were significantly higher than those in the volunteers (Figure 1). The blood concentrations of both markers increased as renal function decreased (Figure 2). This tendency was especially strong for N-ERC.

To confirm that the upward trend in the concentrations of these markers was caused by renal dysfunction and not by other factors (age, sex, diabetes, and hypertension), we examined these factors with multiple regression analysis. The results of this analysis show that renal function strongly influenced the concentrations of these markers, regardless of any effect of the other covariates (Table II).

Table II. *Multivariate analysis of risk factors for the elevation of blood concentration of tumour markers in the CKD group.*

| | N-ERC/mesothelin | | Osteopontin | |
|---|---|---|---|---|
| | F-value | *P*-value | F-value | *P*-value |
| Stage | 52.90 | <0.0001 | 4.76 | 0.0015 |
| HT | 2.00 | 0.16 | 2.20 | 0.15 |
| DM | 1.11 | 0.30 | 0.02 | 0.90 |
| Gender | 0.36 | 0.55 | 0.61 | 0.44 |
| Age | 4.44 | 0.04 | 0.10 | 0.75 |

CKD: chronic kidney disease, DM: diabetes mellitus, HT: hypertension.

## Discussion

Mesothelioma initially progresses along the surfaces of the pleura and peritoneum without forming masses, which means that it is anatomically difficult to diagnose at an early stage with current imaging technologies. Therefore, the need for tumour markers is greater for mesothelioma than for other tumour types. Furthermore, it is important not only to develop high quality tumour markers but also to use them effectively.

Tumour markers can be influenced by many factors, one of which is renal function. At present, there are a number of published reports that suggest a relation between renal function and the blood concentrations of tumour markers (13-22). However, the published data is still very limited and insufficient to allow the knowledge to be put into effective clinical practice. We think it is important to determine how strong this relation is because such information may lead to the development of new and more sensitive diagnostic strategies.

Our study has shown that the concentrations of N-ERC and OPN are influenced by renal function. First, we simply compared the concentrations of N-ERC and OPN from patients with CKD to those of healthy volunteers. In the case of N-ERC, the results show there is a possibility that renal function strongly influences its concentration (Figure 1A, median: 7.144 versus. 3.175 ng/ml for the CKD and control groups, respectively, $p<0.0001$). In the case of OPN, the P-value was statistically significant ($p=0.007$), but the difference in median values was smaller (Figure 1B).

Then, we examined the relation between the degree of renal dysfunction and the concentrations of these markers. The concentration of N-ERC increased as the CKD stage increased (Figure 2), which indicates that there is a strong relation between renal function and the concentration of N-ERC. OPN showed a tendency similar to that of N-ERC, but the tendency was weaker, and the dispersion was wider.

We finally investigated the strengths of the influence of renal dysfunction, diabetes, hypertension, and age on the concentrations of N-ERC and OPN because of the fact that

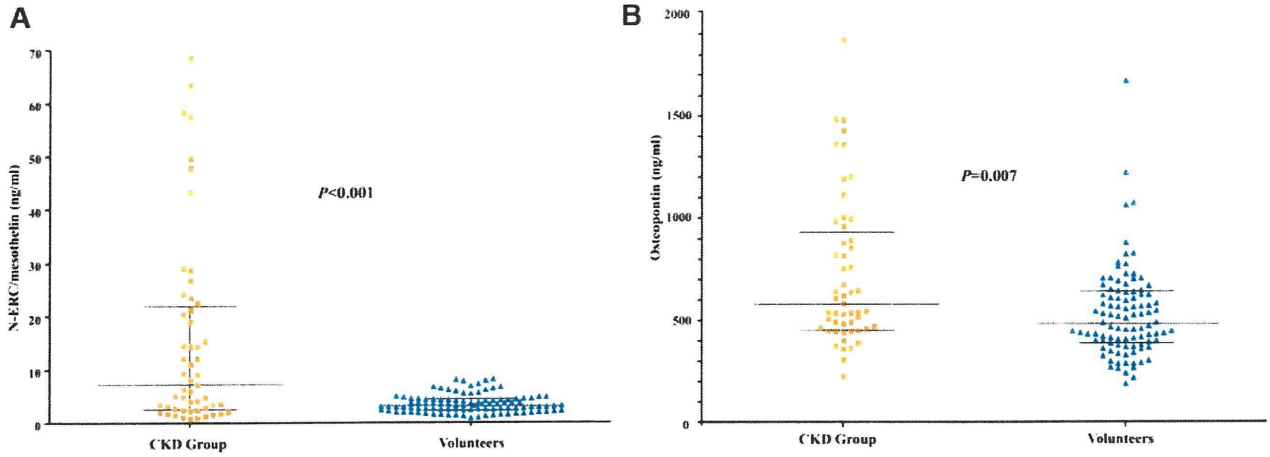


Figure 1. *A: Serum N-ERC/mesothelin levels in the chronic kidney disease group (CKD, comprising renal dysfunction and hemodialysis patients) and healthy volunteers. B: Plasma osteopontin levels of the CKD group and the volunteers. The lines showed 25 percentile, median (50 percentile) and 75 percentile.*

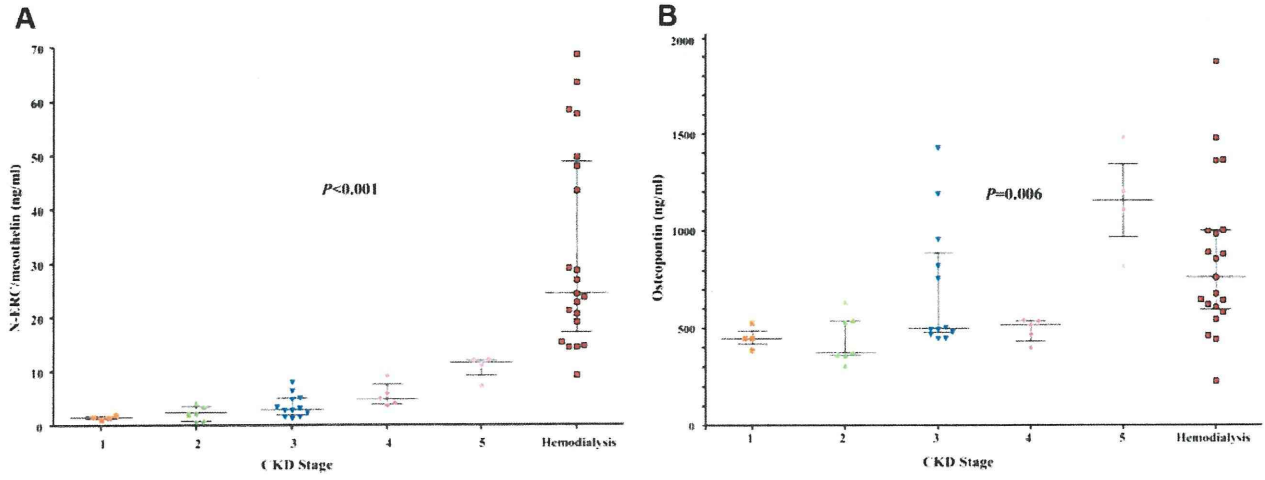


Figure 2. *A: Correlation between chronic kidney disease stage and serum levels of N-ERC/mesothelin. B: Correlation between chronic kidney disease stage and plasma levels of osteopontin.*

more than half of the study patients with CKD had diabetes and hypertension and because our previous data showed serum N-ERC to increase with age (2). Our results supported the hypothesis that renal dysfunction influences the concentrations of these markers. A previous report showed a relation between the tumour marker for mesothelioma, megakaryocyte proliferating factor (MPF), and renal dysfunction (23). Because MPF is the same molecule as N-ERC, which is an N-terminal fragment of the mesothelin molecule, this finding also supports our hypothesis.

Of course, the present study is small and requires further validation. Other factors that are thought to influence the concentration of these markers should be assessed. However, the results of our study show that the consideration of renal function is very important in daily practice and suggests that investigating other factors that have the possibility of affecting the concentration of tumour markers is also important for their effective use.

Our next steps will be to study to what extent diagnostic specificity is increased by developing a new marker that has been adjusted to the severity of renal dysfunction and to investigate in detail whether there are other important diseases that influence the concentrations of N-ERC and OPN. We will also determine the generality of these effects by examining other markers that may be affected by renal dysfunction.

## Conclusion

The concentration of N-ERC/mesothelin appears to be strongly influenced by the degree of renal dysfunction. The concentration of osteopontin is also influenced by renal dysfunction but to a lesser extent than that of N-ERC. The extent of renal failure must be considered when these markers are used in routine practice.

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## References

1 Sugarbaker DJ, Flores RM, Jaklitsch MT, Richards WG, Strauss GM, Corson JM, DeCamp MM Jr., Swanson SJ, Bueno R, Lukanich JM, Baldini EH and Mentzer SJ: Resection margins, extrapleural nodal status, and cell type determine postoperative long-term survival in trimodality therapy of malignant pleural mesothelioma: results in 183 patients. J Thorac Cardiovasc Surg *117*: 54-63, 1999.

2 Shiomi K, Miyamoto H, Segawa T, Hagiwara Y, Ota A, Maeda M, Takahashi K, Masuda K, Sakao Y and Hino O: Novel ELISA system for detection of N-ERC/mesothelin in the sera of mesothelioma patients. Cancer Sci *97*: 928-932, 2006.

3 Shiomi K, Hagiwara Y, Sonoue K, Segawa T, Miyashita K, Maeda M, Izumi H, Masuda K, Hirabayashi M, Moroboshi T, Yoshiyama T, Ishida A, Natori Y, Inoue A, Kobayashi M, Sakao Y, Miyamoto H, Takahashi K and Hino O: Sensitive and specific new enzyme-linked immunosorbent assay for N-ERC/mesothelin increases its potential as a useful serum tumor marker for mesothelioma. Clin Cancer Res *14*: 1431-1437, 2008.

4 Tajima K, Hirama M, Shiomi K, Ishiwata T, Yoshioka M, Iwase A, Iwakami S, Yamazaki M, Toba M, Tobino K, Sugano K, Ichikawa M, Hagiwara Y, Takahashi K and Hino O: ERC/mesothelin as a marker for chemotherapeutic response in patients with mesothelioma. Anticancer Res *28*: 3933-3936, 2008.

5 Hino O and Maeda M: Diagnostic tumor marker of asbestos-related mesothelioma. Environ Health Prev Med *13*: 71-74, 2008.

6 Hagiwara Y, Hamada Y, Kuwahara M, Maeda M, Segawa T, Ishikawa K and Hino O: Establishment of a novel specific ELISA system for rat N- and C-ERC/mesothelin. Rat ERC/mesothelin in the body fluids of mice bearing mesothelioma. Cancer Sci *99*: 666-670, 2008.

7 Maeda M and Hino O: Molecular tumor markers for asbestos-related mesothelioma: serum diagnostic markers. Pathol Int *56*: 649-654, 2006.

8 Maeda M and Hino O: Blood tests for asbestos-related mesothelioma. Oncology *71*: 26-31, 2006.

9 Okio H, Kazu S and Masahiro M: Diagnostic biomarker of asbestos-related mesothelioma: example of translational research. Cancer Sci 98: 1147-1151, 2007.

10 Hino O: Multistep renal carcinogenesis in the Eker (*Tsc 2* gene mutant) rat model. Curr Mol Med *4*: 807-811, 2004.

11 Nakanishi M, Kajino K, Ikesue M, Hagiwara Y, Kuwahara M, Mitani H, Horikoshi-Sakuraba Y, Segawa T, Kon S, Maeda M, Wang T, Abe M, Yokoyama M and Hino O: Establishment of the enzyme-linked immunosorbent assay system to detect the amino-terminal secretory form rat Erc/Mesothelin. Cancer Sci *98*: 659-664, 2007.

12 Tramonti G, Ferdeghini M, Donadio C, Norpoth M, Annichiarico C, Bianchi R and Bianchi C: Renal function and serum concentration of five tumor markers (TATI, SCC, CYFRA 21-1, TPA, and TPS) in patients without evidence of neoplasia. Cancer Detect Prev *24*: 86-90, 2000.

13 Cararach V, Casals E, Martínez S, Carmona F, Aibar C, Quintó LI, Alonso P and Fortuny Abnormal renal function as a cause of false-positive biochemical screening for Down's syndrome. Lancet *350*: 1295, 1997.

14 Tramonti G, Ferdeghini M, Donadio C, Annichiarico C, Norpoth M, Bianchi R and Bianchi C: Tumor-associated trypsin inhibitor (TATI) and renal function. Kidney Int Suppl *63*: S179-S181, 1997.

15 Danişman A, Kiliç S, Kukul E, Yakupoğlu G, Güntekin E, Baykara M and Sevük M: Do renal failure and hemodialysis have any effect on the elimination of free and total prostate-specific antigen? Eur Urol *37*: 579-581, 2000.

16 Nakahama H, Tanaka Y, Fujita Y, Fujii M and Sugita M: CYFRA 21-1 and ProGRP, tumor markers of lung cancer, are elevated in chronic renal failure patients. Respirology *3*: 207-210, 1998.

17 Nomura F, Koyama A, Ishijima M, Takano S, Narita M and Nakai T: Serum levels of five tumor markers for lung cancer in patients with chronic renal failure. Oncol Rep *5*: 389-392, 1998.

18 Eskiocak S, Dortok H, Alvur M and Cengiz K: Tumour markers in chronic renal failure and haemodialysis patients. Nephrol Dial Transplant *10*: 1256, 1995.

19 Cases A, Filella X, Molina R, Ballesta AM, Lopez-Pedret J and Revert L: Tumor markers in chronic renal failure and hemodialysis patients. Nephron *57*: 183-186, 1991.

20 Filella X, Cases A, Molina R, Jo J, Bedini JL, Revert L and Ballesta AM: Tumor markers in patients with chronic renal failure. Int J Biol Markers *5*: 85-88, 1990.

21 Walz G, Kunzendorf U, Josimovic-Alasevic O, Preuschoff L, Schwarz A, Keller F, Asmus G, Offermann G, Diamantstein T and Distler A: Soluble interleukin 2 receptor and tissue polypeptide antigen serum concentrations in end-stage renal failure. Nephron *56*: 157-161, 1990.

22 Odagiri E, Jibiki K, Takeda M, Sugimura H, Iwachika C, Abe Y, Kihara K, Kihara Y, Itou M, Nomura T, Kubo K, Sugino N, Ohta K, Demura R and Demura H: Effect of hemodialysis on the concentration of the seven tumor markers carcinoembryonic antigen, alpha-fetoprotein, squamous cell carcinoma-related antigen, neuron-specific enolase, CA 125, CA 19-9 and CA 15-3 in uremic patients. Am J Nephrol *11*: 363-368, 1991.

23 Hassan R, Remaley AT, Samoson ML, Zhang J, Cox DD, Pingpank J, Alexander R, Willingham M, Pastan I and Onda M: Detection and quantitation of serum mesothelin, a tumor marker for patients with mesothelioma and ovarian cancer. Clin Cancer Res *12*: 447-453, 2006.

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