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シンポジウム 2（腫瘍）難治性卵巣癌の克服を目指して

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To Aim for Conquest the Refractory Ovarian Cancer

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卵巣癌は手術と化学療法との組み合わせによる集学的治療によって，その予後は改善してきた．しかしながら，いまだ予後不良な進行癌，再発癌，化学療法抵抗性癌，特殊組織型癌などの難治性卵巣癌が存在する．これら難治性卵巣癌に対して，初回手術法およびサルベージ療法の検討，至適化学療法の開発，分子標的治療薬の導入，さらにはバイオマーカーの探索による治療の個別化が求められている．

本シンポジウムでは，この視点に立って，難治性卵巣癌に対する新たな治療戦略を見出すための臨床研究や，臨床応用に主眼を置いた基礎的研究を応募し，厳正な審査の結果，以下の 4 名が選ばれた．

1. 板持広明講師（鳥取大学）

卵巣明細胞腺癌では線維芽細胞増殖因子受容体（FGFR）2 遺伝子発現が亢進しており，FGFR2 蛋白が予後予測マーカーとなり得ることを明らかにした．また，FGFR やその下流の PI3K/Akt/mTOR 経路を標的とした新規治療法開発の可能性を示した．

2. 本原剛志助教（熊本大学）

卵巣癌幹細胞の発癌過程における分子機構，癌幹細胞による腹膜播種病巣の形成の機序，そして癌幹細胞とニッチとして機能する骨盤腹膜との相互作用を解明し，“種”としての癌幹細胞と，その“土壌”となる癌幹細胞ニッチを標的とした新規治療戦略の開発を目指した．

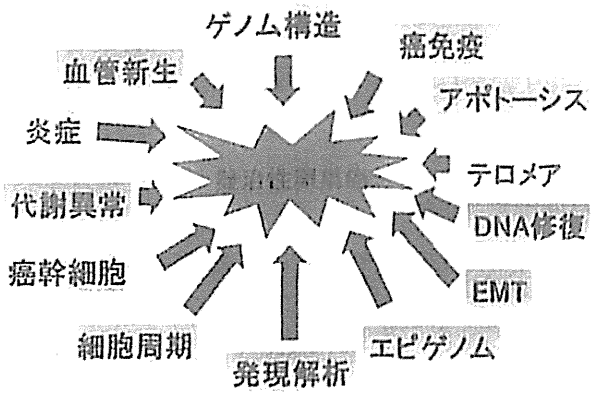
3. 寺井義人准教授（大阪医科大学）

卵巣癌における上皮間葉形態転換（Epithelial-Mesenchymal Transition：EMT）の臨床的意義を明らかにした．細胞表面マーカー CD24 の卵巣癌における発現意義とその機能解析を行い，CD24 を介した EMT のメカニズム解明し，CD24 陽性細胞をターゲットとした臨床応用を目指した治療戦略を目指した．

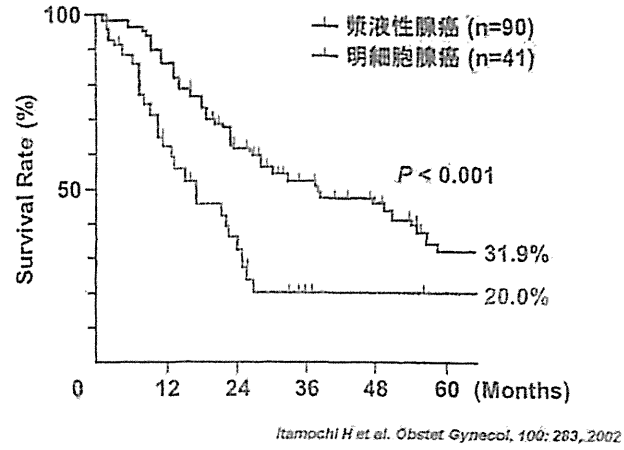
4. 濱西潤三助教（京都大学）

癌細胞は，さまざまな方法で宿主免疫の攻撃か

キーワード：難治性卵巣癌，発現解析，癌幹細胞，上皮間葉形態転換，癌免疫逃避機構
今回の論文に関して，開示すべき利益相反状態はありません．



【図 1】　難治性卵巣癌の分子レベルでの解明と治療戦略



【図 2】　累積生存率(III-IV 期)

ら逃避する能力(癌免疫逃避機構)を獲得している．本研究では，卵巣癌における癌免疫逃避機構を解明し，同機構を標的とした新たな治療戦略を開発し臨床応用によって，有効性を確認した．

本シンポジウムでは「難治性卵巣癌の分子レベルでの解明と治療戦略」を骨子として進行した．1 番目の板持広明先生には「発現解析」，2 番目の本原剛志先生には「癌幹細胞」，3 番目の寺井義人先生は「上皮間葉形態転換」，4 番目の濱西潤三先生には「癌免疫」の切り口から解説していただいた(図 1)．

1. 卵巣明細胞腺癌に対する線維芽細胞増殖因子受容体(FGFR)2 シグナル伝達経路を標的とした新たな治療戦略

鳥取大学医学部生殖機能医学　板持広明

図 2 のごとく，III-IV期明細胞腺癌は漿液性腺癌に比較し，明らかに予後が悪い．その明細胞腺癌に焦点を絞り，「発現解析」の切り口から解説していただいた．

①卵巣明細胞腺癌における特徴的な遺伝子の抽出

卵巣チョコレート嚢胞，卵巣明細胞腺腺，明細胞腺癌検体よりレーザーマイクロダイセクションにより TOTAL RNA を抽出し，遺伝子発現解析を施行し，FGFR2 遺伝子が抽出され，明細胞腺癌において FGFR2 遺伝子の発現増加および増幅を同定した．

②バイオマーカーとしての可能性の検討

卵巣明細胞腺癌 112 例に対し免疫染色を行い，バイオマーカーとしての FGFR2 の可能性を検討している．FGFR2 の蛋白発現は優位に予後と相関していることが示されている．このことから，FGFR2 は予後予測においてバイオマーカーとなりうることが示された．

③新規治療法の開発(FGF 受容体，PI3K/Akt/mTOR)

FGFR 阻害薬である PD173074 は Akt や ERK 1/2 のリン酸化を抑制し，また細胞周期を G1 アレストに導くことが示された．細胞株を用いた実験系では，FGF 受容体阻害薬は新規治療法となりうる可能性が示された．

PI3K/mTOR 阻害薬である BEZ235 は細胞周期を G1 アレストに導くことが示された．

細胞株を用いた実験系では，PI3K/mTOR 阻害薬は新規治療法となりうる可能性が示された．PI3K/mTOR 阻害薬である BEZ235 は，マウスを用いた実験系においても抗腫瘍効果を示した．

以上より本研究の成果は次の 3 点にまとめられる．

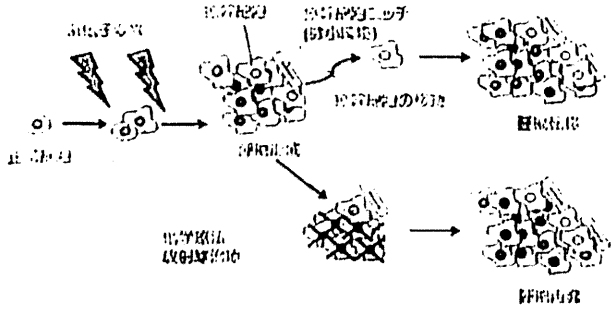
i)卵巣明細胞腺癌において，FGFR2 が高発現していた．

ii)卵巣明細胞腺癌において，FGFR2 は予後予測マーカーとなることが示された．

iii)FGFR やその下流の PI3K/Akt/mTOR 経路を標的とした新規治療法開発の可能性が示され

癌幹細胞：癌組織中、自己複製能を持ち、異なる分化段階の細胞を産生する能力を有する細胞

正常細胞の癌幹細胞化と腫瘍転移および再発のメカニズム



【図3】　癌幹細胞

た．

今後の課題は以下の2点である．

i)FGFRやPI3K/mTOR阻害剤と抗癌剤との併用療法の可能性

ii)FGFR2の下流の経路でPI3K/Akt/mTOR経路以外のシグナル伝達経路の治療標的としての可能性

2. 卵巣癌幹細胞および癌幹細胞ニッチとして機能する骨盤腹膜を標的とした新たな治療戦略

熊本大学大学院生命科学研究部　産科婦人科学分野　本原　剛志

癌幹細胞とは癌組織中，自己複製能を持ち，異なる分化段階の細胞を産生する能力を有する細胞と考えられ，癌幹細胞ニッチとは癌幹細胞を育む微小循環である(図3)．その両者を標的とした治療戦略が示された．

①卵巣癌幹細胞モデルマウスの樹立とEpCAMを標的とした治療戦略

組織幹細胞としての性質を有するEpCAM陽性細胞に対して，遺伝子操作による発癌誘導を行うことで卵巣癌マウスモデルを樹立した．形成されたマウス卵巣腫瘍において，EpCAM陽性の腫瘍細胞が癌幹細胞としての特性(腫瘍形成能，多分化能，Sphere形成能)を有することが示された．また，EpCAM陽性の腫瘍細胞は抗癌剤治療抵抗性に関与することが示された．卵巣漿液性腺癌においてEpCAM高発現症例は有意に予後が悪く，EpCAMを標的とした治療戦略への展開が期待され，カツマキソマブCatumaxomab(anti-EpCAM×anti-CD3)が注目されている．カツマキソマブは，EpCAM陽性の癌細胞およびCD3陽性のTリンパ球に結合し，さらにそのFc断片を介して抗原提示細胞にも結合する3つの機能をもった抗体である．カツマキソマブは，既にEU諸国において治療抵抗性の癌性腹膜炎症例に対して認可されている．現在，医療上の必要性の高い未承認薬・適応外薬の要望として，本薬剤の公知申請を行っている(日本産科婦人科学会)．

②骨盤腹膜において癌幹細胞ニッチとして機能するTAMを標的とした治療戦略

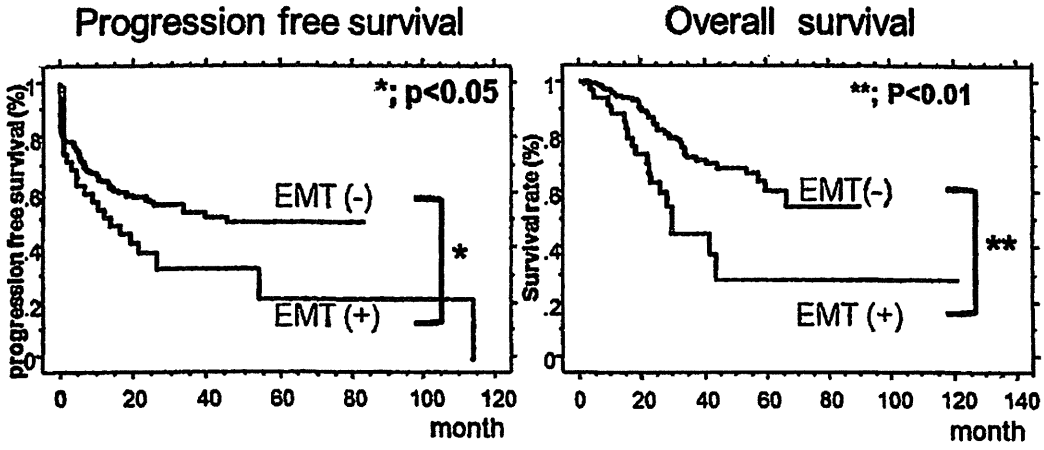
まず，腹膜播種病巣において，CD44variant高発現の症例およびTAMの割合が高い症例は予後不良であることを示した．さらに，癌幹細胞ニッチとして機能するマクロファージが抗癌剤抵抗性に関与していることが示された．これらを踏まえ，卵巣癌の腹膜播種病巣の形成過程において，CD44variant陽性細胞は各種サイトカインの産生およびSTAT3の活性化によって，M2マクロファージとしての性質を示すTAMを周囲に集積させ，自身に最適な微小環境ニッチを形成している可能性を示した．

③骨盤腹膜に存在する癌幹細胞およびニッチを標的とした手術治療戦略

卵巣癌基本術式にpre-metastatic nicheとなり得る骨盤腹膜の広範切除術(WRPP：Wide resection of the pelvic peritoneum)を追加することは，進行卵巣癌症例の予後を改善する可能性がある．WRPPを施行することは，microscopic peritoneal carcinomatosisに存在するEpCAM陽性/CD44variant陽性の卵巣癌幹細胞の切除に有効である可能性を示した．さらに再発率および再発部位の検討を行ったところ，骨盤腹膜への播種病巣として再発した症例が最も高い割合でみられた．WRPP追加群は，スタンダード群と比較して骨盤腹膜への再発が少ない傾向が示された．

以上より本研究の成果は次の2点にまとめられる．

i)卵巣癌幹細胞とマクロファージとの細胞間相互作用の基軸となる骨盤腹膜を重要な再発腫瘍の



【図 4】 EMT 現象と予後

起源の一つであるとした場合，骨盤腹膜を広範囲に切除することは，卵巣癌幹細胞および癌幹細胞ニッチを標的とした治療概念として重要である．

ii)EpCAM および CD44 variant 陽性の卵巣癌幹細胞を標的とした分子標的治療，そして癌幹細胞ニッチとして機能する TAM を標的とした治療が新たな治療戦略として有望であることが示された．

今後の課題は以下の 2 点である．

i)基礎的研究によるマウス発癌モデルの作成によって見出された EpCAM が，ヒト卵巣癌においても重要な役割を担っており，特に抗癌剤抵抗性に関与していることが明らかにされた．今後，EpCAM を標的とした分子標的薬であるカツマキソマブが新たな治療戦略として有望であることが示された．

ii)骨盤腹膜において卵巣癌幹細胞と癌幹細胞ニッチとの細胞間相互作用に関する新たな知見が示されており，今後は腫瘍随伴マクロファージを標的とした多彩な治療戦略への展開が期待される．

3. 難治性卵巣癌の浸潤・転移に関わる分子をターゲットとした新規治療法の開発

大阪医科大学産婦人科学教室　診療准教授　寺井義人

寺井先生は癌細胞が間質内へ浸潤し転移する過程の現象である上皮間葉形態転換(Epithelial-Mesenchymal Transition：EMT)に着目し，卵巣癌における EMT 現象の解明・制御および治療への応用について解説された．

①卵巣癌における EMT の臨床病理学的意義

臨床検体を用いて，Snail 核内染色陽性かつ E-cadherin の減弱を EMT 現象(+)と判定し検討したところ，EMT 現象は卵巣がんにおける浸潤・転移の特徴である腹腔内播種転移を反映していると考えられた．また，卵巣がんにおける EMT 現象は，多変量解析で独立した予後因子であった(図 4)．

②CD24 強発現の臨床病理学的特徴

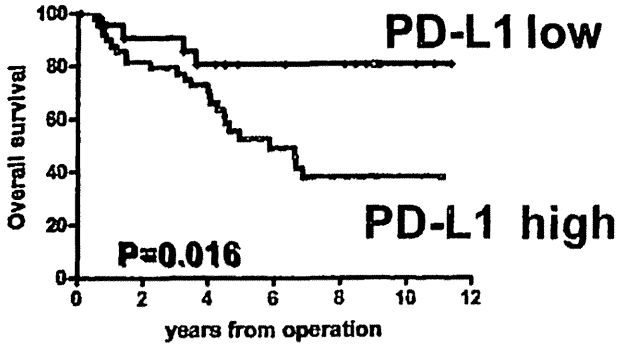
次に，様々な癌腫で過剰発現が予後不良因子であると報告されている CD24 陽性細胞に着目して解析したところ，CD24 強発現は，卵巣癌における腹腔内播種転移，リンパ節転移の有無と有意に相関していた．また，卵巣癌における CD24 強発現は，予後不良因子となり得た．さらに CD24 強発現と EMT 現象との間には有意な相関関係があることが明らかとなった．

③CD24 陽性細胞の特徴

そこで，CD24 陽性細胞の生物学的特性を解析したところ，非足場増殖能が高い，増殖スピードは遅い，浸潤能が亢進している，抗癌剤耐性性が高まる，自己複製能と分化能を有するなどの EMT 現象を起こす細胞の特徴を有した．

④治療への応用

より選択的に薬剤を投与するために，CD24 陽



【図5】

性細胞を認識するシスプラチン内包の高分子ミセルを開発した．CD24陽性卵巣癌細胞を移植したマウスの生存率をPBS群，シスプラチン単独投与群に比べて有意に延長させた．以上よりCD24陽性細胞を認識できる高分子ミセルのDrug delivery systemによる選択的な治療が，難治性卵巣がんに有効である可能性が示唆された．

以上より本研究の成果は，i)癌細胞におけるCD24陽性細胞の存在は，EMTが起きていることを予想させ，predictive markerとなり得る，ii)将来的にCD24陽性細胞をターゲットとした戦略的な治療が検討されることである．

今後の課題として，CD24陽性細胞の本質の解明，高分子ミセルのDrug delivery systemの臨床応用への試みがあげられる．

4. 化学療法抵抗性卵巣癌の局所免疫の解析に基づいた癌免疫逃避を標的とした新規治療戦略

京都大学医学部附属病院　産科婦人科　助教
濱西　潤三

濱西先生は最近注目をされている「癌免疫逃避機構」に着目し，卵巣癌の生物学的特性である，腹膜播種および化学療法抵抗性とがん免疫との関係を解明し新規治療法を開発することを目的とした研究について解説された．

①卵巣癌における癌免疫と免疫逃避機構

マウス卵巣癌腹膜播種モデルにて，腫瘍内へのCD8＋T細胞の誘導は予後改善に寄与する可能性があり，実際の臨床検体において，CD8T細胞浸潤が多い症例は，予後良好であった．一方で，免疫抑制因子であるPD-L1の発現は，予後不良因子であった(図5)．

②PD-L1発現機序と腹膜播種に及ぼす影響

そこで，腫瘍のPD-L1発現メカニズムおよび腹膜播種に及ぼす影響について検討した．卵巣癌のPD-L1発現は腹膜播種と関連し，このPD-L1発現は腹水中のリンパ球が産生するIFNγによって誘導された．また，卵巣癌のPD-L1発現はCD8＋T細胞機能を抑制し腹膜播種を促進したが，一方でPD-L1発現を抑制すると，T細胞性免疫が活性化し，さらに生存期間も有意に延長したことから，PD-L1/PD-1経路は，卵巣癌腹膜播種の治療標的として有望であると考えられた．

③化学療法が癌免疫に及ぼす影響

化学療法は，卵巣癌細胞にNF-kB経路活性化を介して，PD-L1発現を誘導し，癌微小環境の免疫状態を変化させていた．さらに化学療法と同時にPD-1/PD-L1経路を阻害すると抗腫瘍効果が増強することが示唆された．

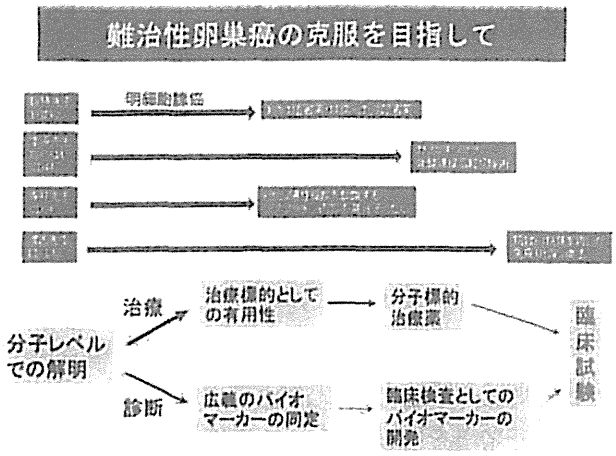
以上から，現行のTC療法に加え，抗PD-1抗体を併用するChemo-Immunotherapyは，卵巣癌治療の新規治療戦略として有望であることが示唆された．

④医師主導型臨床治験とバイオマーカー検索

PD-1・PD-L1シグナルの阻害抗体により免疫を再活性化できることが知られており，臨床応用がすすめられている．濱西らはプラチナ抵抗性再発・進行卵巣癌患者を対象に完全ヒト型抗PD-1抗体(Nivolumab)を用いた治療法の有効性および安全性を評価することを目的とした医師主導型臨床治験を2011年9月より開始した．本年3月に目標症例20例の登録が終了し，現在解析中である．

以上より本研究の成果は，i)卵巣癌においてPD-L1発現による免疫逃避機構が明らかとなり，ii)抗PD-1抗体(Nivolumab)は再発例など従来の化学療法では治療効果の期待できない患者に治療の道が開けたことである．

今後の課題は適応症例を同定するバイオマーカーの検索および検証である．



【図6】 難治性卵巣癌の克服を目指して

まとめ

卵巣癌の治療抵抗性の獲得には，図1のように多くの要因が関与している．本シンポジウムでは，発現解析，癌幹細胞，EMT，癌免疫をキーワードに4人の演者にそれぞれの要因の難治性卵巣癌の病態への関与とそれを克服するための治療戦略を提示していただいた．基礎研究にとどまらず，それぞれが臨床応用への道筋を示していただき，明るい展望が感じられた(図6)．しかし，難治性卵巣癌の克服のためには，これ以外の要因に対する戦略も必要である．我々はさらに病態に関与する分子レベルの解明を進め，それを基に新規治療法を開発する努力を続け，患者の予後の改善につなげていくことが大切である．

癌幹細胞の同定と治療への応用

楠木総司*1　加藤聖子*2　竹田　省*1

癌幹細胞の同定

胚性幹細胞以外に正常の組織の中に自己増殖能や多分化能を持つ組織幹細胞が存在するといわれているが，近年癌幹細胞という概念が提唱されている。癌幹細胞の定義としては①自己複製能を有する②様々なphenotypeの腫瘍細胞から構成された腫瘍そのものを再生することができる③腫瘍細胞のなかでもわずかにしか存在しない④造腫瘍能を持たない細胞にも分化する多分化能を持つ⑤特異的な分化マーカーを発現する，といった特徴を満たすものである。現行の癌治療では癌幹細胞を排除することはできず，生き残った癌幹細胞が新たに腫瘍を形成し，再発や転移に寄与すると考えられる。

癌幹細胞を分離する手段としてCD133やCD44，ALDH1などの癌幹細胞特有のマーカーを用いてフローサイトメトリー(fluorescence activated cell sorting；FACS)で分離・同定する方法のほかに，DNA結合色素Hoechst33342で細胞を染色し，幹細胞が発現するABCトランスポーターを介して，色素を細胞外に排出することを利用してside population(SP)細胞という細胞集団を分離，同定する方法がある。

われわれは子宮体癌細胞Hec1細胞株よりSP細胞を分離し，Hec1SP細胞の生物学的特性を解析し，分化マーカー発現の低下，長期増殖能，自己複製能の獲得，造腫瘍能亢進などの特性を示すことを明らかにし，子宮体癌細胞株Hec1細胞由来のSP細胞(Hec1-SP細胞)は癌幹細胞様の性質を示すことを報告している[1]。

癌幹細胞とEMT

Epitherial mesenchymal transition(EMT)は上皮細胞が間葉系細胞に形態変化する現象であり，初期胚発生において重要な役割をしている。また，癌細胞が転移し多臓器に浸潤する際にもEMTを起こすと考えられている。2008年にマニらは，EMTを起こした細胞は幹細胞様の性質を持つということを報告した[2]。SnailとtwistのようなEMTを起こす遺伝子を導入した乳がん細胞は，$CD44^{high}/CD24^{low}$という乳癌幹細胞の細胞集団が増え，上皮のマーカーの発現が低下し，fibronectinやvimentinのような間葉系マーカーの発現が上昇し，EMTを誘導した乳癌幹細胞はEMTの性質を持つことを報告している[2]。

われわれもmicroarrayを用いて，ラット子宮内膜細胞にヒト*K-RAS*癌遺伝子を導入したRK12V細胞のSP細胞とnon-SP(NSP)細胞間で発現量に差がみられた上位10個のパスウェイのうち，EMTに関与するパスウェイが4つ認められた。また，Hec1-SP，-NSP細胞間で，EMT関連遺伝子の発現量をReal-time PCRで比較したところfibronectinの発現比が高く，子宮体癌幹細胞の特性にEMTが関与することを確認した[3]。

癌幹細胞を標的とした治療

イオノフォア抗生物質であるサリノマイシンがEMTの性質を持つ乳癌幹細胞の増殖を特異的に阻害することが報告された[4]。その実験では，サリノマイシン投与により，タキソール投与の100倍以上の乳癌幹細胞の増殖抑制効果がみられた。サリノマイシンの癌幹細胞の増殖抑制効果は多くの研究で示されているが，子宮体癌幹細胞に対する効果については報告がなかった。そこでFACSを用いた幹細胞の分離・同定を行っている東京医科歯科大学難治疾患研究所幹細胞制御分野の田賀哲也教授との共同研究で，子宮体癌細胞株からSP細胞を分離し解析を進めていくことになったのが本研究の発端である。

Hec1-SP細胞の増殖能・運動能(scratch assay)・浸潤能(Boyden chamber assay)に対するサリノマイシンの効果を検討し，その抑制効果を確認した。また，サリノマイシン添加時の細胞形態は核の膨化とクロマチンの断片化がみられ，Tunnel法を用いてアポトーシスが関与していることを確認した。また最近の報告では，サリノマイシンは*LEF1*，*cyclin D1*や*fibronectin*などの*Wnt*シグナルの標的遺伝子の発現を抑制するといわれており，Real-time PCRで*LEF1*，*cyclin D1*の発現を調べたところ，サリノマイシン投与により発現抑制効果がみられサリノマイシンがWntシグナルを阻害する可能性が示唆された。

また*in vivo*におけるHec1-SP細胞の造腫瘍能におけるサリノマイシンの抑制効果についても確認している[3]。

おわりに

癌幹細胞を標的とした治療としては癌幹細胞で高発現している遺伝子群を同定し，それを標的とした抗体を用いた薬剤の開発が進められているが，薬剤スクリーニングによる癌幹細胞を特異的に攻撃する薬剤の同定はサリノマイシンが初めての報告であろう。*In vitro*での効果は乳癌だけでなく，肺癌，大腸癌，膵臓癌，前立腺癌，白血病などでも報告されており，様々な癌種の癌幹細胞を標的とした治療として有望な薬剤であると考えられるが，サリノマイシンはその毒性のため，ヒトへの投与はできない。現在，ヒトへの応用を目指して，誘導体を開発中の海外製薬会社との共同研究を進めている。

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共同研究施設　研究内容紹介

東京医科歯科大学難治疾患研究所幹細胞制御分野は，2008年に熊本大学発生医学研究センター(現，熊本大学発生医学研究所)より異動，設立された研究室である。生体組織の形成と維持・再生を司る「幹細胞」を研究対象として，増殖分化因子群や接着因子群などを介した細胞外来性シグナルと，エピジェネティック修飾などの細胞内在性プログラムなど，多角的な視点から幹細胞制御の分子基盤を解明することを目標としている。

私たちが研究対象としている幹細胞の1つに「癌幹細胞」がある。癌幹細胞は腫瘍組織の発生と維持・再発を司る中心的細胞であることから，癌根絶の絶好の標的として有力視され，その実態は徐々に明らかになっている。近年は，癌幹細胞とそれを維持する微小環境(ニッチと称される)との相互作用を阻止することで癌幹細胞の駆逐を図る「ニッチ標的療法」の概念も提唱されている。

私たちはエジンバラ大学との共同研究で，癌幹細胞のニッチを模倣する合成ポリマーのスクリーニングを行い，癌幹細胞画分として定義されるSP(side population)画分にいまだ多様性が存在することを見出した。また国立がん研究センター研究所との共同研究では，この人工ニッチポリマーの表面に結合するいくつかのニッチ候補分子を同定することができた。同様のスクリーニングシステムは正常組織幹細胞にも適用可能であり，ニッチ研究を遂行する上で強力な解析ツールとなることが示唆される。

この章に紹介されている順天堂大学との共同研究をはじめ，これまでに子宮癌や卵巣癌などいくつかの婦人科腫瘍において癌幹細胞の存在は示唆されている。また，不妊症や子宮内膜症など種々の産婦人科疾患においても，正常組織幹細胞とそのニッチの分子基盤の解明が病因・病態の理解と新規治療法・診断法の開発へと繋がることが期待される。(楠　順一，田賀哲也)

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Research Article

A Disintegrin and Metalloproteinase 9 Is Involved in Ectodomain Shedding of Receptor-Binding Cancer Antigen Expressed on SiSo Cells

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In several human malignancies, the expression of receptor-binding cancer antigen expressed on SiSo cells (RCAS1) is associated with aggressive characteristics and poor overall survival. RCAS1 alters the tumor microenvironment by inducing peripheral lymphocyte apoptosis and angiogenesis, while reducing the vimentin-positive cell population. Although proteolytic processing, referred to as "ectodomain shedding," is pivotal for induction of apoptosis by RCAS1, the proteases involved in RCAS1-dependent shedding remain unclear. Here we investigated proteases involved in RCAS1 shedding and the association between tumor protease expression and serum RCAS1 concentration in uterine cancer patients. A disintegrin and metalloproteinase (ADAM) 9 was shown to be involved in the ectodomain shedding of RCAS1. Given the significant correlation between tumor ADAM9 expression and serum RCAS1 concentration in both cervical and endometrial cancer as well as the role for ADAM9 in RCAS1 shedding, further exploration of the regulatory mechanisms by which ADAM9 converts membrane-anchored RCAS1 into its soluble form should aid the development of novel RCAS1-targeting therapeutic strategies to treat human malignancies.

1. Introduction

To date, over 150 scientific reports have been published that concern the biological functions and clinical significance of RCAS1. RCAS1 is a 639 amino acid, type II membrane protein with an N-terminal transmembrane segment and a C-terminal coiled-coil structure that is involved in oligomer formation [1]. Since RCAS1 promotes tumor cell evasion of immune surveillance by inducing apoptosis in immune cells, including peripheral lymphocytes, and also remodels the cancer stromal microenvironment, RCAS1 is believed to contribute to tumor progression [2]. Clinically, RCAS1 expression is significantly higher in cancerous tissues relative to normal tissues [3], and its expression increases during the progression from precancerous lesions to cancer [4, 5]. RCAS1 expression is associated with several clinicopathological parameters of human malignancies, including histological type, differentiation, tumor size, stage, depth of invasion, lymphovascular space involvement, lymph node metastasis, and positive peritoneal cytological results [6]. In addition, RCAS1 is a negative predictor of overall survival in 15 different kinds of cancers occurring in the brain, oral cavity, lung, pleural mesothelium, esophagus, stomach, bile duct, gallbladder, pancreas, colon, gastrointestinal mesenchyme, kidney, prostate, uterine cervix, and endometrium [2].

RCAS1 is shed in the serum and pleural effusion and as such may be a useful biomarker for human cancer due to its ability to predict the results of medical treatments [7, 8]. During the conversion from a membrane-anchored to a shedded protein, RCAS1 undergoes proteolytic processing known as "ectodomain shedding" [9]. Ectodomain shedding affects the biological activity of membrane proteins such as growth factors, growth factor receptors, cell-adhesion molecules, and extracellular matrix proteins by altering their localization and mode of action [10]. For membrane-anchored growth factors, ectodomain shedding can convert them into diffusible factors and greatly influence their functions. The membrane-anchored form of Spitz, a transforming growth

factor (TGF)-α-like molecule that is an epidermal growth factor receptor (EGFR) ligand in *Drosophila*, is inactive but is activated following proteolytic cleavage to yield a soluble protein [11]. In contrast, membrane-anchored c-kit ligand [12] and ephrins [13] are fully functional, while their soluble forms exhibit little or no biological activity. RCAS1 induces apoptosis mainly via its shedded form and not the membrane-anchored form. Therefore, regulation of the conversion of membrane-anchored proteins into their soluble form would be an important way to modify the action of these molecules, including RCAS1. Accumulating evidence demonstrated a role for proteolytic enzymes such as matrix metalloproteinase (MMP), ADAM, and the closely related ADAM with thrombospondin motifs (ADAMTSs) in cancer development and progression [14, 15]. MMPs, ADAMs, and ADAMTSs play a crucial role during all stages of cancer progression, from initiation to metastatic spreading. Besides their role in shedding of plasma membrane-associated proteins and intracellular signaling, these proteases regulate growth factor activation, angiogenesis, inflammation, and apoptosis [16, 17].

Although RCAS1 induces apoptosis mainly after being converted to its shedded form, the proteases involved in this ectodomain shedding remain unclear. To understand more clearly the regulation of membrane-anchored RCAS1 conversion, we sought to (1) identify key proteases involved in RCAS1 shedding and (2) determine whether there is an association between tumor protease expression and serum RCAS1 concentration in cervical and endometrial cancer patients.

2. Materials and Methods

2.1. Cell Lines. The human uterine cervical adenocarcinoma cell line SiSo, human breast adenocarcinoma cell line MCF-7, human chronic myelogenous leukemia cell line K562, and mouse embryo fibroblast L cells were maintained in RPMI 1640 medium supplemented with 100 units/mL penicillin G, 100 μg/mL streptomycin, and 10% fetal bovine serum (FBS) (ICN Biomedical, Irvine, CA) in a humidified incubator (37°C, 5% CO_2). Both SiSo and MCF-7 cells express membrane-anchored RCAS1, but RCAS1 shedding is undetectable in MCF-7 culture supernatants [9]. RCAS1 expression and shedding are undetectable in K562 cells and L cells that express a putative RCAS1 receptor [1]. We established the SiSo cell line from uterine cervical adenocarcinoma [18] and the other cell lines were purchased from the American Type Culture Collection.

2.2. Patients and Surgical Specimens. Tissue samples from cervical and endometrial cancer patients were used for immunohistochemical analysis. All patients had received medical treatment between April 2010 and January 2013 at the Department of Obstetrics and Gynecology, Kyushu University Hospital (Table 1). The mean patient age was 43 years (range of 24–81 years) for cervical cancer and 58 years (range of 37–84 years) for endometrial cancer. The histologic subtypes were 33 cases of squamous cell carcinoma and 14 cases of adenocarcinoma in cervical cancer and 48 cases of endometrioid adenocarcinoma (24 cases of grade 1; 15 cases of grade 2; 9 cases of grade 3) in endometrial cancer. Cases were classified into stages as follows: 36 cases: stage I; 11 cases: stage II in cervical cancer; 28 cases: stage I; 5 cases: stage II; 13 cases: stage III; 2 cases: stage IV in endometrial cancer. These specimens were graded according to the 2008 International Federation of Gynecology and Obstetrics criteria. All specimens were fixed, embedded in paraffin, and stained with hematoxylin and eosin for determination of histologic subtype. Informed consent was obtained from all patients in this study. This study protocol was approved by the Ethical Committee of Kyushu University.

Table 1: Clinicopathologic variables for uterine cancer patients.

Clinicopathologic variables	Number of patients
Cervical cancer	
Age (years; mean ± SD)	43 ± 11
Stage	
I	36
II	11
Histologic subtype	
Squamous cell carcinoma	33
Adenocarcinoma	14
Endometrial cancer	
Age (years; mean ± SD)	58 ± 11
Stage	
I	28
II	5
III	13
IV	2
Grade	
1	24
2	15
3	9

2.3. Evaluation of RCAS1 Expression by Flow Cytometry. To evaluate RCAS1 expression, flow cytometric analysis was performed using the monoclonal antibody 22-1-1 (MBL, Nagoya, Japan) that recognizes human RCAS1. Briefly, cells were harvested followed by incubation with 22-1-1 antibody on ice for 45 minutes. After the cells were washed, they were incubated for 45 minutes with fluorescein isothiocyanate-conjugated goat anti-mouse IgM antibody (Pierce, Rockford, IL) on ice. The cells were again washed, and flow cytometric analysis was performed using FACScan (Becton Dickinson, San Jose, CA).

2.4. Enzyme-Linked Immunosorbent Assay (ELISA). We measured RCAS1 concentrations of the cell culture supernatant in triplicate with an ELISA kit (MBL, Nagoya, Japan), according to the manufacturer's instructions. The RCAS1 ELISA kit was applied in earlier investigations of the clinical significance of RCAS1 in uterine cancer [19]. The sensitivity of the RCAS1

assay was 0.008 U/mL. Mean concentrations of triplicate measurements were calculated.

2.5. Generation of SiSo Cells Expressing ADAM9 Small Interfering Ribonucleic Acid (siRNA). To construct specific siRNA for ADAM9, oligonucleotides were synthesized and purified by Takara Bio (Shiga, Japan) as follows: sense 5′-GGAGAUUUGGACCAAUGGATT-3′ and antisense 5′-UCCAUUGGUCCAAAUCUCCTT-3′. The target specificity of these sequences was confirmed by a BLAST search (http://www.ncbi.nlm.nih.gov/gene/). Homologous siRNA oligonucleotides were dissolved in buffer (100 mM potassium acetate, 30 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) plus potassium hydroxide, 2 mM magnesium acetate, pH 7.4) to a final concentration of 20 μM, heated to 90°C for 60 seconds, and incubated at 37°C for 60 minutes before use to disrupt higher order aggregates formed during synthesis. The complexes of transfection reagent (Invitrogen, Carlsbad, CA) plus siRNA were added to SiSo culture dishes. Assays were performed 48 hours after treatment. A nontargeting control siRNA that did not have homology with known gene targets in mammalian cells was also used. The control siRNA GC content was 38.1%, which is identical to that of the siRNAs constructed here.

2.6. Generation of MCF-7 Cells Stably Expressing ADAM9. MCF-7 cells were transfected with the expression vector pEF-BOS carrying human ADAM9 complementary deoxyribonucleic acid (cDNA) or an empty pEF-BOS vector using Lipofectamine 2000 reagent (Invitrogen). Transfected cells were selected with 250 μg/mL G418. A clone was established after transfection with ADAM9 cDNA that was named MCF-7/ADAM9. One clone was isolated after transfection with the pEF-BOS vector alone.

2.7. Western Blot Analysis. Cells were lysed in radioimmunoprecipitation assay buffer (1% Triton-X, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 150 mM NaCl, 50 mM Tris (pH 8.0), 0.2 unit/mL aprotinin, 2 μg/mL leupeptin, 1 μg/mL pepstatin A, 2 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate). Extracts were then subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting analysis after transfer to Immobilon-P transfer membranes (Millipore Corporation, Bedford, MA). Membranes were probed with several antibodies including rabbit anti-ADAM9 (Chemicon, Temecula, CA) and mouse anti-β-actin (Novus Biologicals, Littleton, CO) antibodies. Peroxidase-conjugated goat anti-rabbit (Southern Biotech, Birmingham, AL) or anti-mouse IgG (Chemicon) was used as a secondary antibody.

2.8. Microarray Analysis. Hybridization targets for the GeneChip Human Gene 1.0 ST array were prepared using the GeneChip WT cDNA Synthesis and Amplification Kit, GeneChip Sample Cleanup Module and GeneChip WT Terminal Labeling Kit according to the manufacturer's protocol (Affymetrix, Santa Clara, CA). Briefly, the cells were harvested in the logarithmic growth phase and total RNA was extracted. Total RNA (100 ng) was converted into double-stranded cDNA (1st-cycle), and the complementary RNA (cRNA) was synthesized by *in vitro* transcription. After purification and measurement of cRNA, 10 μg was converted into single-stranded DNA (ssDNA, 2nd cycle), of which 5.5 μg was fragmented and labeled. The ssDNA was hybridized to the array described above for 16 hours at 45°C. Following hybridization, the array was automatically washed and stained with the GeneChip Hybridization, Wash and Stain Kit. The Probe Array was scanned using the GeneChip Scanner 3000 7G. Microarray analysis was performed three times using three independent cell cultures.

2.9. Evaluation of Apoptotic Cell Death. Induction of apoptosis in K562 cells was evaluated by coculturing with four effecter cell types, including SiSo, MCF-7, MCF-7/ADAM9, or L cell. Each effecter cell (1×10^5 cells/well) and K562 target cells were coincubated in a 6-well plate at 1–20 : 1 effecter/target (E/T) ratio. To enhance tight cell-cell contacts, the plates were centrifuged once after coculture initiation. The suspended cells were harvested and stained with the Annexin V-PE apoptosis detection kit (MBL) on days 1–4 after beginning the experiment. Flow cytometric analysis was performed to measure the number of apoptotic cells. To discriminate K562 cells from effecter cells, K562 cells were stained using the green fluorescence cell linker PKH kit (Sigma, St. Louis, MI) before coculture initiation. Evaluation of K562 cell apoptosis was performed three times using three independent cell cultures.

2.10. Immunocytochemical Detection of RCAS1 and ADAM9 Association. A proximity ligation assay (PLA) to detect an association between RCAS1 and ADAM9 was carried out using a Duolink detection kit (Olink Bioscience, Uppsala, Sweden). Briefly, SiSo, MCF-7, ADAM9 siRNA-transfected SiSo, and RCAS1 siRNA-transfected SiSo cells [20] were seeded into 8-well chamber slides. On the next day, cultures were fixed in 90% ethanol/5% acetic acid and subjected to PLA. Slides were incubated with mouse anti-RCAS1 (MBL) and rabbit anti-ADAM9 (Chemicon) antibodies and then secondary antibodies conjugated to unique DNA probes (PLA probe MINUS and PLUS) were added. Ligation and circularization of the DNA were followed by a rolling circle amplification step, and reactions were detected by a complementary Tex613 fluorophore-labeled DNA linker [21]. Slides were evaluated using an LSM 510 META confocal microscope (Zeiss, Jena, Germany).

2.11. Immunohistochemistry. For immunohistochemical analyses, one or two representative samples selected for each case were analyzed by means of the streptavidin-biotin method. The 22-1-1 antibody (MBL) or rabbit anti-ADAM9 antibody (Abcam, Cambridge, MA) was applied as the primary antibody. Positive control samples were as follows: for RCAS1, cervical adenocarcinoma, which was used to manufacture the 22-1-1 antibody [3], and for ADAM9, breast cancer [22]. We also performed assays without immunized mouse and rabbit immunoglobulins as negative controls. No

Table 2: Microarray data on proteases.

Gene symbol	Probe ID	Probe set ID	SiSo signal	MCF-7 signal	SiSo/MCF-7
ADAM2	HU133p2_17106	207664_at	7.0	6.3	1.11
ADAM3A	HU133p2_26379	217090_at	1.4	1.9	0.74
ADAM5	HU133p2_26289	216998_s_at	2.9	7.2	0.40
ADAM6	HU133p2_47159	237909_at	3.1	7.1	0.44
ADAM7	HU133p2_20597	211239_s_at	7.3	3.0	2.43
ADAM8	HU133p2_14627	205179_s_at	12.3	8.4	1.46
ADAM9	HU133p2_11830	202381_at	1856.6	275.1	6.75
ADAM10	HU133p2_06424	1562137_at	5.2	4.7	1.11
ADAM11	HU133p2_49087	239837_at	9.6	8.2	1.17
ADAM12	HU133p2_51710	242460_at	9.2	6.4	1.44
ADAM15	HU133p2_26298	217007_s_at	83.6	43.4	1.93
ADAM17	HU133p2_22834	213532_at	117.7	195.5	0.60
ADAM18	HU133p2_17039	207597_at	5.6	8.3	0.67
ADAM19	HU133p2_30412	221128_at	8.9	13.3	0.67
ADAM20	HU133p2_16866	207423_s_at	7.7	18.7	0.41
ADAM21	HU133p2_17107	207665_at	6.8	6.2	1.10
ADAM22	HU133p2_53445	244194_at	5.9	5.6	1.05
ADAM23	HU133p2_15493	206046_at	6.2	24.0	0.26
ADAM28	HU133p2_17694	208269_s_at	1.6	7.4	0.22
ADAM29	HU133p2_30621	221337_s_at	0.4	9.1	0.04
ADAM30	HU133p2_30730	221446_at	3.7	9.5	0.39
ADAM32	HU133p2_00020	1552266_at	11.3	10.4	1.09
ADAM33	HU133p2_43119	233868_x_at	33.1	31.9	1.04
ADAMTS1	HU133p2_31443	222162_s_at	0.6	4.4	0.14
ADAMTS2	HU133p2_23835	214535_s_at	3.6	2.3	1.57
ADAMTS3	HU133p2_24209	214913_at	4.5	5.3	0.85
ADAMTS4	HU133p2_02285	1555380_at	13.9	16.1	0.86
ADAMTS5	HU133p2_29220	219935_at	2.2	2.4	0.92
ADAMTS6	HU133p2_09800	1570351_at	3.5	8.2	0.43
ADAMTS7	HU133p2_29991	220706_at	2.2	5.9	0.37
ADAMTS8	HU133p2_29962	220677_s_at	8.4	12.4	0.68
ADAMTS9	HU133p2_03368	1556989_at	6.4	11.1	0.58
ADAMTS10	HU133p2_41388	232133_at	1.2	0.8	1.50
ADAMTS12	HU133p2_30705	221421_s_at	13.3	19.8	0.67
ADAMTS13	HU133p2_33121	223844_at	3.0	10.1	0.30
ADAMTS15	HU133p2_00845	1553427_at	2.0	1.5	1.33
ADAMTS16	HU133p2_46339	237089_at	2.2	2.4	0.92
ADAMTS17	HU133p2_00332	1552725_s_at	9.9	17.0	0.58
ADAMTS18	HU133p2_00702	1553234_at	9.1	10.7	0.85
ADAMTS19	HU133p2_00663	1553180_at	4.8	26.0	0.18
ADAMTS20	HU133p2_30002	220717_at	2.4	4.6	0.52
ADAMDEC1	HU133p2_15581	206134_at	0.5	4.5	0.11
ADAMTSL1	HU133p2_33638	224371_at	1.0	2.1	0.48
ADAMTSL2	HU133p2_16076	206629_at	1.2	1.2	1.00
ADAMTSL3	HU133p2_04979	1559748_at	2.2	12.0	0.18
MMP1	HU133p2_13923	204475_at	35.0	46.0	0.76
MMP2	HU133p2_08387	1566677_at	1.2	0.4	3.00
MMP3	HU133p2_15276	205828_at	19.3	25.0	0.77

Table 2: Continued.

Gene symbol	Probe ID	Probe set ID	SiSo signal	MCF-7 signal	SiSo/MCF-7
MMP7	HU133p2_13707	204259_at	17.9	5.4	3.31
MMP8	HU133p2_16774	207329_at	0.6	0.4	1.50
MMP9	HU133p2_13384	203936_s_at	21.2	43.1	0.49
MMP10	HU133p2_15128	205680_at	1.1	10.1	0.11
MMP11	HU133p2_45158	235908_at	2.3	14.4	0.16
MMP12	HU133p2_14028	204580_at	3.0	3.9	0.77
MMP13	HU133p2_15407	205959_at	7.9	10.5	0.75
MMP14	HU133p2_09936	160020_at	18.6	28.6	0.65
MMP15	HU133p2_53134	243883_at	1.2	1.6	0.75
MMP16	HU133p2_17594	208166_at	3.7	4.7	0.79
MMP17	HU133p2_15681	206234_s_at	6.9	28.0	0.25
MMP19	HU133p2_14022	204574_s_at	2.3	4.7	0.49
MMP20	HU133p2_17041	207599_at	1.9	0.9	2.11
MMP21	HU133p2_00237	1552592_at	3.8	0.8	4.75
MMP23A/B	HU133p2_16565	207118_s_at	1.4	0.6	2.33
MMP24	HU133p2_17809	208387_s_at	5.0	5.7	0.88
MMP25	HU133p2_49304	240054_at	14.4	34.8	0.41
MMP26	HU133p2_29826	220541_at	4.1	7.7	0.53
MMP27	HU133p2_30068	220783_at	1.1	0.7	1.57
MMP28	HU133p2_33479	224207_x_at	14.8	23.3	0.64
MMPL1	HU133p2_16735	207289_at	8.7	9.6	0.91

significant immunohistochemical reaction occurred in the control sections.

Immunohistochemical expression of RCAS1 and ADAM9 was reviewed without knowledge of the clinicopathologic data. Evaluation of expression consisted of an examination of five representative fields, with 1000 tumor cells (200 for each field) being counted via a microscope with a high-power (400x) objective. Tissue sections with more than 5% reactive cells were defined as positive and graded as follows: 1+, 5% to 25% positive cells; 2+, 26% to 50% positive cells; and 3+, 51% to 100% positive cells.

2.12. Statistical Analysis. The Fisher's exact (chi-square) test was done to evaluate the association between RCAS1 and ADAM9 expression in tumor tissues resected from uterine cancer patients. The Mann-Whitney test was performed to check differences in antigen expression and secretion between different groups of cells. P values of <0.05 were considered statistically significant.

3. Results

3.1. Differences in Protease Expression between SiSo and MCF-7 Cells. The expression of proteases was compared between SiSo and MCF-7 cells by microarray analysis (Table 2). The ADAM9 expression level was significantly higher in SiSo cells, as shown by relative signals of 1856.6 and 275.1 in SiSo and MCF-7 cells, respectively, which yields a relative ratio of 6.75. No other proteases showing strong expression signals were significantly different between SiSo and MCF-7 cells.

3.2. Changes in RCAS1 Expression and Shedding after Gene Transfection. ADAM9 expression was knocked down in SiSo cells with siRNA. ADAM9 siRNA-transfected cells showed suppressed ADAM9 expression and inversely increased RCAS1 expression on the cell surface (Figure 1(a) (A) (B)). The RCAS1 expression and concentration were also quantitatively analyzed and shown in Figure 1(a) (C). While transfection of ADAM9 siRNA significantly augmented RCAS1 expression, the amount of RCAS1 in the culture supernatant was markedly decreased ($P = 0.0495$). On the other hand, ADAM9 expression was upregulated in MCF-7 cells following transfection of ADAM9 cDNA (Figure 1(b) (A)). However, RCAS1 expression was significantly reduced, even though RCAS1 shedding was accelerated by induction of ADAM9 expression (Figure 1(b) (B) (C)) ($P = 0.0495$). On the other hand, the ADAM17 expression level was also higher than other proteases. ADAM17 is expressed in various tissues and has been reported to be associated with cancer progression events such as invasion, migration, and metastasis [15]. We also evaluated RCAS1 expression and shedding after ADAM17 gene transfection and found no significant change in either expression or shedding (see Supplementary Figure 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2014/482396). Taken together, these data indicate that ADAM9 rather than ADAM17 is involved in RCAS1 shedding.

3.3. Analysis of Apoptotic Cell Death Induced in K562 Cells. Apoptosis of K562 cells was induced using a coculture system

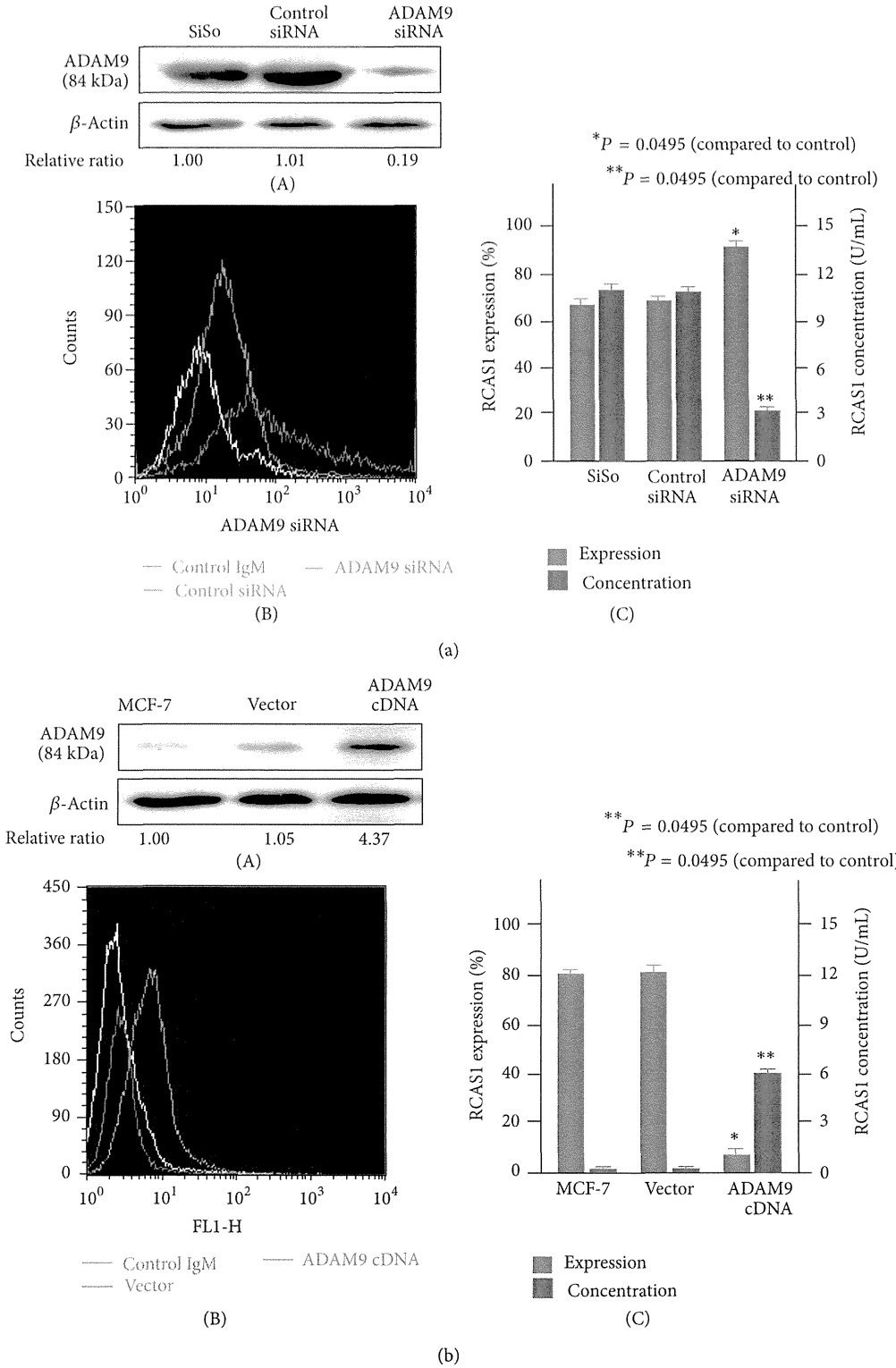

FIGURE 1: Change in RCAS1 expression and shedding after gene transfection. (a) ADAM9 siRNA transfection in SiSo cells. (A) A Western blot revealed that ADAM9 expression diminished after ADAM9 siRNA transfection. (B) Flow cytometric analysis showed that cell surface expression of RCAS1 increased after ADAM9 siRNA transfection. (C) Transfection of ADAM9 siRNA significantly augmented RCAS1 expression but decreased the RCAS1 concentration in culture supernatants ($P = 0.0495$). (b) ADAM9 cDNA transfection in MCF-7 cells. (A) A Western blot revealed that ADAM9 expression was enhanced after ADAM9 cDNA transfection. (B) Flow cytometric analysis showed that the cell surface expression of RCAS1 decreased after ADAM9 cDNA transfection. (C) Transfection of ADAM9 cDNA significantly diminished RCAS1 expression but increased the concentration of RCAS1 in culture supernatants ($P = 0.0495$). Mean values of triplicate measurements are shown.

with SiSo, MCF-7, MCF-7/ADAM9, and L cells. Both L cells and MCF-7 cells were used as a negative control and SiSo cells were a positive control for inducing apoptosis in K562 cells [9]. Although K562 apoptosis was not induced by coculture with MCF-7 cells, the effecter cell MCF-7/ADAM9 could induce apoptosis in K562 cells. Figure 2(a) shows that the annexin-V positive ratio increased dependently on the E/T ratio after 4 days of culture wherein 22.5% (this number represents the percentage of cells that were double positive for PKH2 and annexin-V) of K562 cells were apoptotic with a 20 : 1 E/T ratio. K562 apoptosis increased significantly depending on the culture period (Figure 2(b)) ($P = 0.0495$).

Next, we measured the RCAS1 concentration in cell supernatants. Although RCAS1 was not detected in L cells and MCF-7 cells, MCF-7/ADAM9 cells shed RCAS1 depending on the length of the culture period (Figure 2(c)). The level of RCAS1 shed from MCF-7/ADAM9 cells was lower than for SiSo cells, but the amount of RCAS1 significantly increased after four days of culture as compared to the first day ($P = 0.0495$). These results suggest that apoptosis of K562 cells was induced by RCAS1 that was shed after ADAM9 proteolysis.

3.4. RCAS1 and ADAM9 Expression in Cell Lines and Cancerous Tissues. RCAS1 and ADAM9 colocalization was immunocytochemically analyzed in SiSo and MCF-7 cells using a Duolink detection kit. Negative controls without immunized immunoglobulins showed no red dots indicating colocalization of RCAS1 and ADAM9 (Supplementary Figures 2(a) and 2(b)). While red dots were observed in SiSo cells, they were absent in MCF-7 cells (Figure 3(a) (A) (B)). Although red dots in ADAM9 siRNA-transfected SiSo cells and RCAS1 siRNA-transfected SiSo cells rarely occurred (Figure 3(a) (C) (D)), they were occasionally observed in MCF-7/ADAM9 cells (Supplementary Figure 2(e)).

RCAS1 and ADAM9 expression was also evaluated in cancerous tissues by immunohistochemistry. ADAM9 expression was detected in normal cervical epithelium and endometrial glands with weak cytoplasmic staining and membrane immunoreactivity (data not shown). On the other hand, prominent staining for ADAM9 was detected in cervical and endometrial cancer (Figure 3(b)). The difference in ADAM9 protein expression levels between normal epithelium and cancerous tissues was highly significant. Diffuse staining for RCAS1 and ADAM9 was observed both in the cytoplasm and on the cell membrane of cancer cells. Of 47 patients with cervical cancer, 8, 17, 13, and 9 cases showed no expression, 1+, 2+, and 3+ of RCAS1, respectively, while 7, 8, 11, and 21 cases had no expression, 1+, 2+, and 3+, respectively, for ADAM9. Two cases were double-negative and 34 cases were double-positive for RCAS1 and ADAM9. Five cases were single-positive for RCAS1 and 6 cases were single-positive for ADAM9. In 48 patients with endometrial cancer, 16, 14, 12, and 6 cases showed no expression, 1+, 2+, and 3+, respectively, for RCAS1, while for ADAM9, 6, 9, 14, and 19 cases had no expression, 1+, 2+, and 3+, respectively. Two cases were double-negative and 28 cases were double-positive for RCAS1 and ADAM9. Four cases were single-positive for RCAS1 and 14 cases were single-positive for ADAM9. There was no statistically significant association between RCAS1 and ADAM9 expression in both cervical and endometrial cancer.

3.5. The Association between Serum RCAS1 Concentration and RCAS1/ADAM9 Expression in Uterine Cancer Patients. We evaluated the association between serum RCAS1 concentration and RCAS1/ADAM9 expression in 47 cervical and 48 endometrial cancer patients. Serum RCAS1 levels significantly increased in a manner that was dependent on RCAS1 and ADAM9 expression in both cancer types (Figures 4(a) and 4(b)). These data further support a role for ADAM9 in regulating RCAS1 shedding in human uterine cancer.

4. Discussion

This is the first report showing that ADAM9 is involved in RCAS1 ectodomain shedding. ADAM9 is a member of the ADAM protein family, for which 40 gene members have currently been identified with 21 members being functional in humans [23]. ADAMs are membrane-anchored glycoproteins that consist of pro- and metalloprotease, disintegrin, cysteine-rich, EGF-like, and cytoplasmic domains, which enable these proteins to have a versatile range of physiological and pathological functions [24]. Some ADAMs participate in fertilization, myogenesis, neurogenesis, and activation of growth factors/immune regulators such as tumor necrosis factor (TNF)-α [25]. On the other hand, specific ADAMs have been implicated in a number of diseases, including rheumatoid arthritis, Alzheimer's disease, atherosclerosis, asthma, and cancer [17, 26]. ADAM9 was cloned and sequenced by Weskamp et al. in 1996 [27]. ADAM9 is widely expressed in the human body and is a catalytically active metalloprotease-disintegrin protein that has been implicated in the ectodomain cleavage of heparin-binding (HB)-EGF and as an α-secretase for the amyloid precursor protein [17]. Olson et al. demonstrated the reproductive stage-specific expression of ADAM9 mRNA in rabbit uterine epithelium during the peri-implantation period [28]. ADAM9 expression is upregulated as progesterone levels rise and at blastocyst implantation sites. ADAM9 also plays a pivotal role in some signaling pathways, wherein transmission of information might induce some inconvertible exacerbations of disease [29]. ADAM9 is reportedly involved in several human diseases such as inflammatory disorders, oxygen-induced retinopathy (OIR), and cancer [30]. ADAM9 expression was found to be upregulated in various solid tumors and is often associated with adverse prognostic parameters or shorter patient survival times. ADAM9 overexpression was reported in several human carcinomas, including oral [31], lung [32], breast [21, 33], stomach [34], liver [35], pancreas [36], colon [37], kidney [38], prostate [39], cervix [40], and melanoma [41], and is correlated with cancer progression and metastasis, as well as having a predictive capacity for patient survival times. The background for the clinical significance of ADAM9 in tumor progression has been investigated by *in vitro* experiments. ADAM9 expression was found to be elevated in a cell line having high metastatic potential as compared to cell lines that had a low metastatic potential [35].

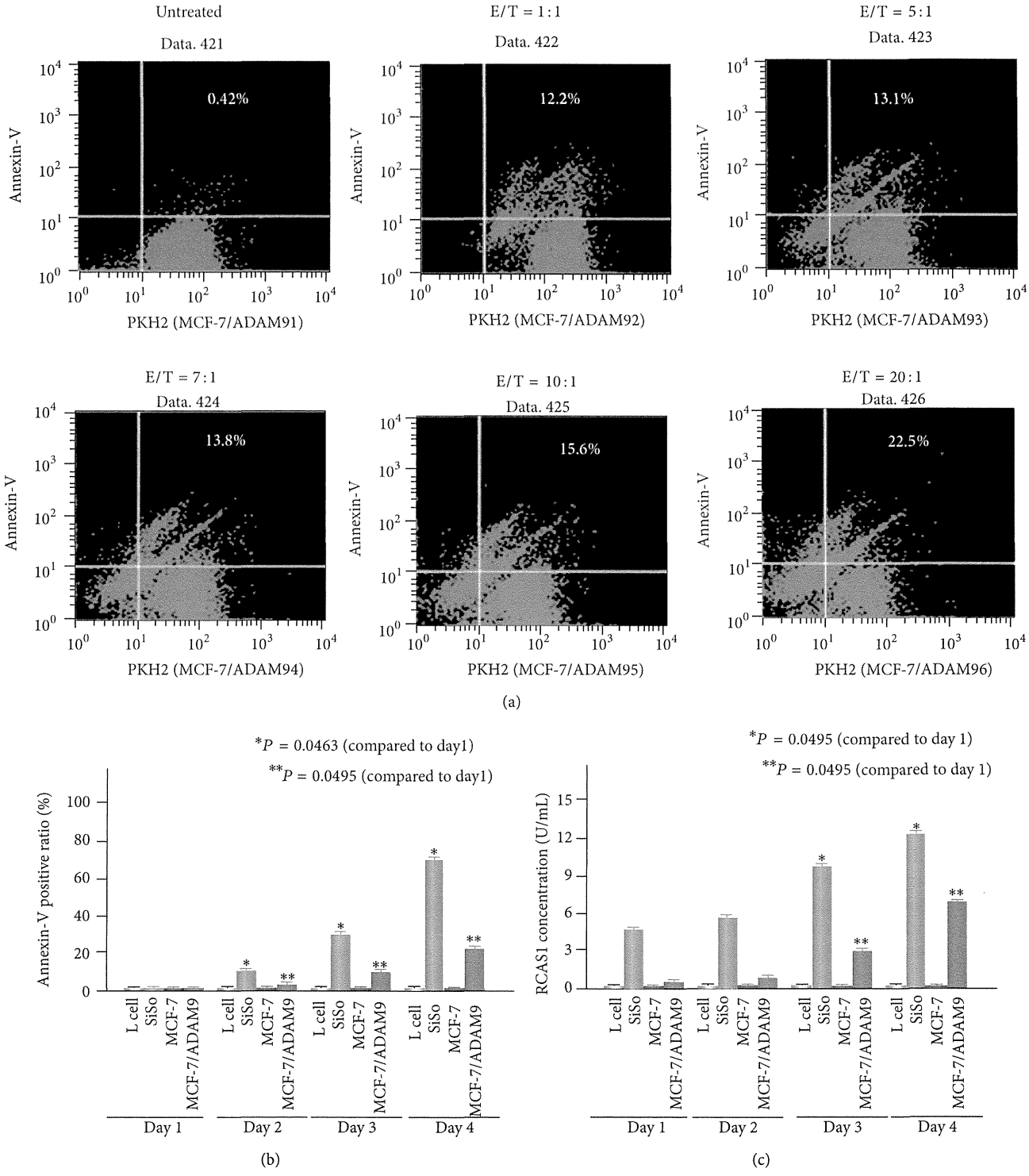

FIGURE 2: Analysis of apoptotic cell death induced in K562 cells. (a) K562 cell apoptosis was analyzed by flow cytometry. Increases in the annexin-V positive ratio were dependent on the E/T ratio after 4 days of culture. The percentage of cells double positive for PKH2 and annexin-V is indicated. (b) The increase in the number of apoptotic K562 cells was dependent on the culture period (E/T ratio = 20 : 1). Both L cells and MCF-7 cells were used as a negative control and SiSo cells acted as a positive control for inducing apoptosis in K562 cells. (c) The RCAS1 concentration in cell supernatants was measured by ELISA. The RCAS1 level in MCF-7/ADAM9 supernatants increased with the culture time, even though MCF-7 cells alone do not shed RCAS1. Mean values of triplicate measurements are shown.

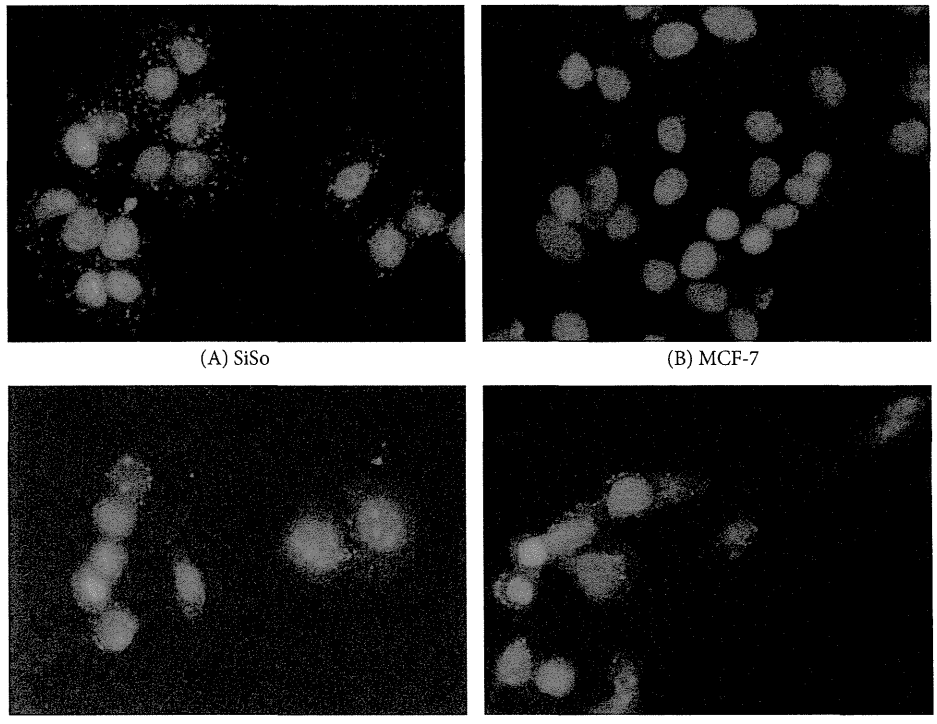

FIGURE 3: RCAS1 and ADAM9 expression in cell lines and cancerous tissues. (a) RCAS1 and ADAM9 colocalization was immunocytochemically analyzed in SiSo, MCF-7, ADAM9 siRNA-transfected SiSo, and RCAS1 siRNA-transfected SiSo cells using the Duolink detection kit. The red dots indicate RCAS1 and ADAM9 colocalization. (b) The expression pattern of RCAS1 and ADAM9 is shown in one representative case from cervical and endometrial cancer. Diffuse staining for RCAS1 and ADAM9 was observed both in the cytoplasm and on the cell membrane of the cancer cells.

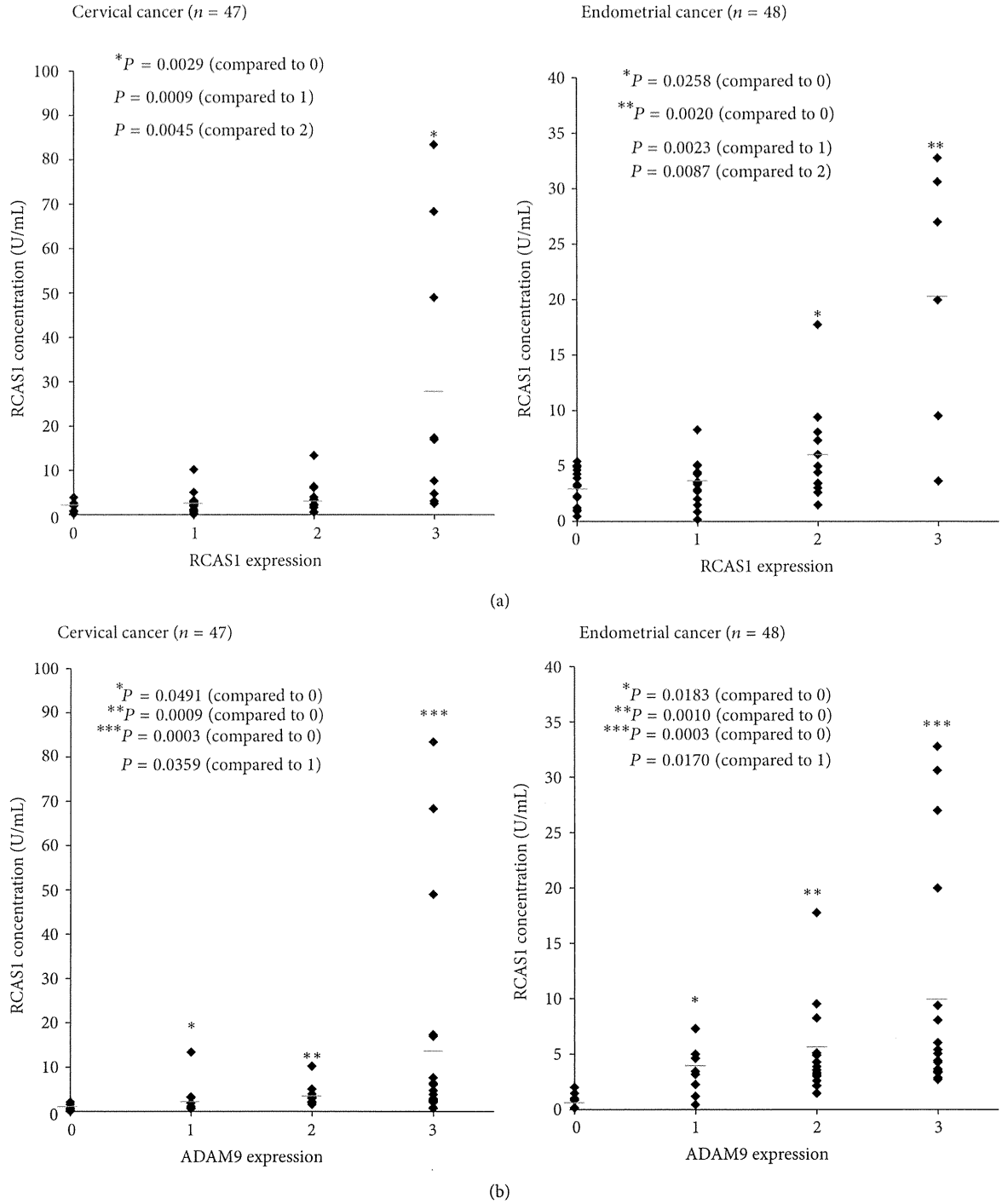

FIGURE 4: Association between serum RCAS1 concentration and RCAS1/ADAM9 expression in uterine cancer patients. Serum RCAS1 levels significantly increased in a manner that was dependent on both RCAS1 (a) and ADAM9 (b) expression in cervical and endometrial cancer patients. Mean values of triplicate measurements are shown.

Enhanced ADAM9 expression induced by gene transfection also promoted cell invasion [37]. ADAM9 is a secreted protein and its soluble form promoted the invasive phenotype of carcinoma cell lines by binding to the $\alpha 6\beta 4$ and $\alpha 2\beta 1$ integrins on the surface of carcinoma cells through its disintegrin domain [42]. In melanoma and a hepatic metastatic site of colon cancer, ADAM9 expression was upregulated at the invasion front, again supporting its role in tumor progression [41, 42].

Zubel et al. previously reported that ADAM9 is expressed in cervical squamous cell carcinoma [40]. In this study, the positive ratio of ADAM9 was 87% (29 out of 33 cases) and

78% (11 out of 14 cases) in squamous cell- and adenocarcinoma, respectively, which are values that are somewhat lower than the 93% (13 out of 14 cases) given in the previous report. On the other hand, this is the first report concerning ADAM9 expression in endometrial cancer wherein ADAM9 positivity was seen in 87% of cases (42 out of 48 cases) and ADAM9 expression was strong (3+) in 19 of 48 cases.

Although cancer patients currently receive multidisciplinary therapies that integrate surgery, radiation, and chemotherapy, limitations in the efficacy of anticancer treatments against advanced or recurrent tumors require the development of novel and highly specific targets for therapy. Considering the significance of tumor progression, RCAS1 has potential value as a unique biomarker and molecular target for diagnostics and therapy. Several therapeutic strategies should thus be considered to suppress the expression and function of RCAS1. A first strategy could be to modulate RCAS1 expression using siRNA. The value of this technique was shown in studies where the induction of molecular-specific siRNA into tumor cells reduced T lymphocyte apoptosis and VEGF secretion, which was followed by tumor regression [8, 43]. A second strategy for inhibiting RCAS1 function is to use antibodies. Serum from uterine and ovarian cancer patients inhibited growth of RCAS1 putative receptor expressing K562 cells, and this suppressive effect could be partially negated after immunoprecipitation to remove RCAS1 [19, 44]. A third strategy would be to modulate ADAM9-mediated RCAS1 ectodomain shedding. Soluble RCAS1, rather than the membrane-anchored form, is mainly responsible for inducing apoptosis [9]. In this study, we did not use a catalytic inactive mutant form of ADAM9 to demonstrate that the proteolytic activity of ADAM9 is directly responsible for RCAS1 shedding. Thus it is possible that ADAM9 indirectly controls RCAS1 processing. To date, several methods have been reported to suppress ADAM9 expression and function. Knockdown of ADAM9 by RNA interference resulted in reduced cell proliferation, invasion, and metastasis [35, 39, 45], as well as increased sensitivity to radiation and chemotherapeutic drugs [46]. Blocking of ADAM9 activity with specific antibodies resulted in inhibited cell growth of gastric cancer cell lines [34]. Reactive oxygen species (ROS) can also induce expression of ADAM9 via p38 mitogen-activated protein kinase activation [47]. Sung et al. observed apoptotic cell death in prostate cancer cells by decreasing ADAM9 expression via the administration of an antioxidant or genetic transfer of a hydrogen peroxide degradative enzyme [39]. Moreover, Moss et al. described that the metalloproteinase inhibitor marimastat is potent against ADAM9 [48].

Before initiating molecular targeting therapy, selection of eligible patients is necessary. For cervical and endometrial cancer in which RCAS1 is a clinical prognostic factor [2], tissue sampling and expression analysis of RCAS1 and ADAM9 can be easily performed. While several selective synthetic inhibitors that are active against a small number of ADAMs have recently been described [49, 50], adverse effects induced by targeting therapy can be a significant concern when the targeted molecules are ubiquitously expressed. Weskamp et al. generated mice lacking ADAM9 to learn more about the function of this protein during development and in adults [51]. During mouse development, ADAM9 mRNA is ubiquitously expressed, with particularly high expression levels in the developing mesenchyme, heart and brain. Despite the ubiquitous expression of ADAM9, these knockout mice appear to develop normally, are viable and fertile, and have no major pathological phenotypes compared to wild-type mice. Therefore, potential adverse effects produced by targeting ADAM9 activity could be anticipated to be tolerable.

Some recent advances might offer in the near future the opportunity to design such specific inhibitors using, for example, siRNAs or monoclonal antibodies. The precise understanding of the exact role played by RCAS1 and ADAM9 in cancer appears to be of particular importance from the perspective of designing new therapeutic strategies that are based on the control or inhibition of these proteins.

5. Conclusion

In several human malignancies, RCAS1 expression is associated with aggressive characteristics and poor overall survival. Since RCAS1 promotes tumor cell evasion of immune surveillance by inducing apoptosis in immune cells and also remodels the cancer stromal microenvironment, RCAS1 is believed to contribute to tumor progression. Soluble RCAS1, rather than the membrane-anchored form, is mainly responsible for inducing immune cell apoptosis. ADAM9 is involved in RCAS1 ectodomain shedding; therefore, inhibition of ADAM9 activity might contribute to controlling the biological functions of RCAS1. A precise understanding of the role played by RCAS1 and ADAM9 is essential to design novel strategies to treat cancer.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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