1. 免疫療法の標的としてのがん細胞亜集団

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がんはさまざまな要因により亜集団をつくるheterogeneousな細胞群である．がん細胞の亜集団による多様性が，治療抵抗性獲得などの新たな形質獲得に寄与する．がんを免疫療法の標的ととらえる場合，がん細胞亜集団に対しても有効な免疫応答を惹起することが免疫療法を成功させるキーになる．がん細胞亜集団としては，がん幹細胞，EMTを起こしているがん細胞，飢餓状態によるオートファジーを起こしているがん細胞などがある．本稿において，がん細胞亜集団が免疫学的にどのように影響するか述べる．

はじめに

がんはheterogeneousであり不均一な細胞集団である．不均一ながん細胞亜集団は，がん幹細胞※1，EMT※2を起こす細胞，オートファジーを起こす細胞などとして知られる（**図1**）．がんは内因的な要因あるいは外因的な要因により，可逆的あるいは非可逆的に亜集団をつくる．亜集団をつくるがん細胞は，母集団とは異なる遺伝子発現を示すために，母集団と異なる形質を獲得する．母集団とは異なり亜集団にのみ発現する遺伝子産物は亜集団特異的抗原分子として免疫の標的になりうる．つまり，がん細胞亜集団は，がん細胞母集団と異なるタンパク質を発現するために亜集団を形成することができ，反面，免疫学的側面からは同タンパク質は特異的な抗原となることが多い．このように亜集団が特異的な抗原分子を発現する場合，亜集団は母集団と比べて免疫学的に免疫原性が高いと考えられる．しかしながら，臨床的ながんでは亜集団が混在

[キーワード＆略語]
がん幹細胞，EMT，オートファジー，CTL

CTL：cytotoxic T lymphocyte（細胞傷害性T細胞）
EMT：epithelial-mesenchymal transition（上皮間葉移行）
MHC：major histocompatibility complex（主要組織適合遺伝子複合体）
MMP：matrix metalloproteinase（マトリックスメタロプロテアーゼ）
PSA：puromycin-sensitive aminopeptidase

※1　がん幹細胞

がん幹細胞は，がん細胞のなかでも①高い造腫瘍能を有する，②自己複製能を有する，③多分化能を有する亜集団，と定義される．がん幹細胞は化学療法や放射線療法といった既存のがん治療法に抵抗性を示すことが知られており，治療後の再発や遠隔転移といった患者予後を直接左右するイベントに深くかかわっていると考えられている．

※2　EMT

がん細胞は胚発生時に発現する転写因子群を使って間葉系形質を獲得する．すなわち，Snail，Slug，Twist，Goosecoid，FOXC2などの転写因子により，E-カドヘリン，デスモゾームの消失，細胞遊走，間質浸潤に必要な分子群を誘導し，上皮細胞由来のがんが，間葉系細胞形質を獲得し，間質に浸潤する．

Cancer subpopulation as an immunotherapeutic target
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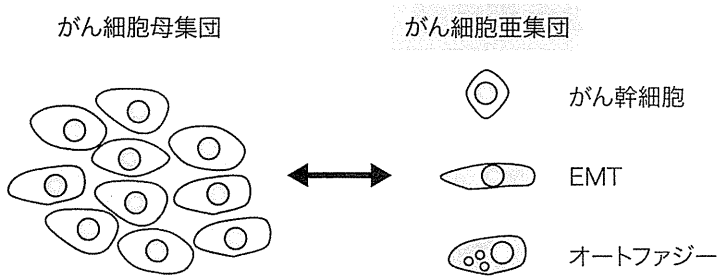

図1　がん細胞亜集団
がんは大部分を構成するがん細胞母集団，および少部分を構成するがん細胞亜集団からなる不均一な細胞集団である．がん細胞亜集団としてがん幹細胞，EMTを起こす細胞，オートファジーを起こす細胞などがある

する．すなわち，発現する抗原分子が多く免疫原性が高いと考えられるがん細胞亜集団は背景に免疫機構から逃避する術を獲得していることが多い（**図2**）．以下，がん細胞亜集団を免疫学的に免疫機構に正に働く抗原分子，および，負に働く免疫逃避の二側面から記載する．

1 がん幹細胞が形成する細胞亜集団

がん幹細胞は化学療法や放射線療法といった治療に抵抗性を示すことが知られている．治療抵抗性を示すがん幹細胞ではあるが，さまざまな免疫エフェクターに対して感受性を示すことが明らかとなっている．獲得免疫エフェクターである細胞傷害性T細胞（CTL）※3，抗体，および，自然免疫を司るエフェクター細胞であるナチュラルキラー（NK）細胞，γδT細胞ががん幹細胞を認識することが報告されている．一方では，がん幹細胞は免疫逃避機構を備えているとの報告もある．

1）がん幹細胞の抗原性

CTLは抗原分子由来の抗原ペプチドを特異的に認識するエフェクター細胞で，獲得免疫のなかでも細胞性免疫を司る細胞である．CTLが標的細胞を認識するうえでは，標的細胞がMHC class I分子に提示された抗原分子由来の抗原ペプチドを発現する必要がある．がん幹細胞は，非がん幹細胞と同等のMHC class I分子発現を示すとの報告，および非がん幹細胞より発現が低いとの報告がある[1)2)]．しかしながら，後者の報告でもがん幹細胞は一定レベルのMHC class I分子が発現すると結論づけており，がん幹細胞はMHC class I分子を発現する亜集団と考えることができる．

CTLが認識する抗原分子の発現は，がん幹細胞および非がん幹細胞での発現様式から，がん幹細胞に優位に発現する抗原（がん幹細胞抗原），がん幹細胞および非がん幹細胞に発現する抗原（共通抗原），非がん幹細胞に優意に発現する抗原（非がん幹細胞抗原）の3グループに分類できる[3)]．がん幹細胞をCTLで標的とする場合は，少なくともCTLが認識する抗原分子ががん幹細胞に発現する必要があり，がん幹細胞抗原もしくは共通抗原となる．共通抗原であるCEP55特異的なCTLを用いて検討した結果，がん幹細胞および非がん幹細胞ともにCEP55特異的CTLに同レベルで認識された[1)]．この結果は，がん幹細胞および非がん幹細胞ともに，CTLに対して感受性を示すことを示唆する．つまり，がん幹細胞はMHC class I分子を発現し，一部の抗原分子（がん幹細胞抗原および共通抗原）を発現し，がん幹細胞抗原および共通抗原を用いればCTLでがん幹細胞を認識できる．

では，免疫療法の標的分子としてがん幹細胞抗原および共通抗原ではどちらがよいのだろうか．がん幹細胞抗原では，がん幹細胞特異的な免疫応答を誘導し，共通抗原では，がん全体を認識する免疫応答誘導が可能となる．マウス遺伝子ワクチンの実験モデルでは，共通抗原分子であるサバイビンを標的とした場合と，

※3　CTL
細胞傷害性T細胞（CTL）は主に，$CD3^+CD8^+$T細胞群で，標的細胞表面のMHC class I分子に提示される抗原ペプチドを認識すると，細胞傷害性顆粒（cytotoxic granule）を分泌する．細胞傷害性顆粒にはパーフォリン，グランザイムが含まれており，パーフォリンが標的細胞膜上で重合し孔をあける．セリンプロテアーゼに属するグランザイムが孔を通じて標的細胞に侵入し，アポトーシスを誘導する．

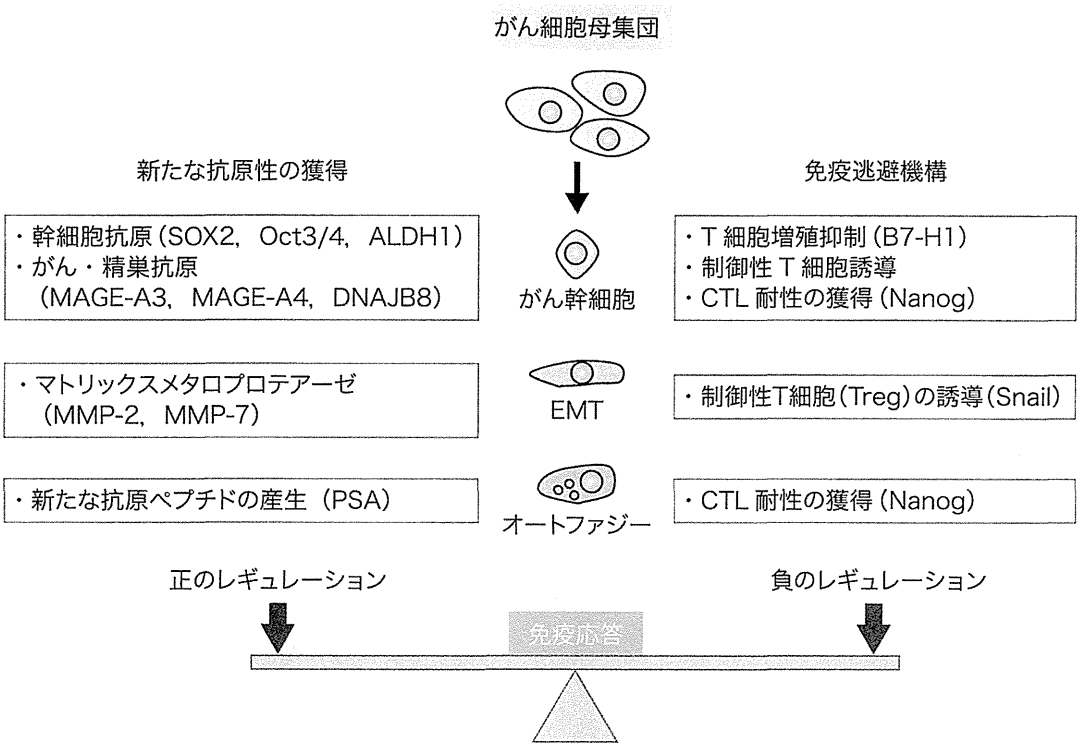

図2　がん細胞亜集団の免疫学的特性
がん細胞亜集団は，がん細胞母集団と比較して，新たな抗原分子を発現し，新たな抗原性を有する．一方では，がん細胞亜集団はさまざまな免疫逃避機構を備え，新たな抗原性を獲得しているにもかかわらず，免疫システムから逃避している

がん幹細胞抗原であるDNAJB8を標的とした場合では，DNAJB8の方が抗腫瘍効果が高かった[4]．このことは，がん幹細胞抗原の方が，共通抗原より高い抗腫瘍効果をもたらす可能性を示唆する．Weissmanらのグループは，新規悪性黒色腫幹細胞マーカーとしてCD271を報告しているが，$CD271^+$悪性黒色腫幹細胞は，ペプチドワクチンの標的となるチロシナーゼ，MART-1，MAGE-C1などの抗原分子の発現が低いことを報告している．われわれは，チロシナーゼやMART-1を標的とした悪性黒色腫免疫療法の治療効果が限定的であるのは，造腫瘍能が高い悪性黒色腫幹細胞にはチロシナーゼやMART-1の発現が低く，免疫療法で悪性黒色腫幹細胞を標的できないことが原因だと考察している[5]．いずれにしても，がん免疫療法で有効な治療効果を得るには，がん幹細胞も標的とできることが必要条件の1つと考えられる．

がん幹細胞抗原としては，さまざまな抗原分子が報告されている．それらの分子は主にがん幹細胞と精巣に発現する，いわゆるがん・精巣抗原（MAGE-A3，MAGE-A4，DNAJB8など）と，がん幹細胞および正常幹細胞に発現する幹細胞共通抗原（SOX2，Oct3/4，ALDH1A1など）に分類できるようである．がん・精巣抗原はMAGE-A3のペプチドワクチンおよびタンパク質ワクチンで臨床効果がみられ，期待される分子群であるが，これらのがん・精巣抗原は，がん幹細胞分画に優位に発現する[6]．がん・精巣抗原の比較的有効な臨床試験の結果は，そのことが理由の1つかもしれない．がん免疫療法の標的分子として非常に期待される分子群である．一方では，幹細胞抗原分子で免疫を惹起した場合，正常幹細胞を傷害し自己免疫疾患を誘発する可能性があり，臨床試験に移行するのは倫理的に問題がある．

前記のとおり，NK細胞やγδT細胞ががん幹細胞を認識することも知られている．NK細胞は，抗原分子非特異的に標的細胞を認識する，自然免疫エフェクターの1つである．Bottinoらのグループは，ヒトグリオーマ組織から分離したグリオーマ幹細胞はナイーブなNK細胞には耐性を示したが，IL-2もしくはIL-15

で活性化したNK細胞に感受性を示したと報告している[7]．ヒト大腸がん幹細胞においても同様の報告がある[2]．大腸がん幹細胞はゾレドロン酸で活性化したVγ9Vδ2T細胞に認識されることも報告されている[8]．NK細胞もγδT細胞もCTL同様，パーフォリンおよびグランザイムを用いて標的細胞を傷害する．これらの報告は，がん幹細胞がCTLをはじめとする免疫細胞に感受性を示すことを支持する．

エフェクター細胞以外にも液性免疫エフェクターである抗体ががん幹細胞を傷害することも報告されている．Weissmanらのグループは，急性骨髄性白血病（AML）幹細胞には，正常の造血幹細胞同様に膜タンパク質であるCD47が発現することを報告している．CD47はマクロファージの抑制性受容体であるSIRPαを介して，マクロファージの貪食作用を抑制する．造血幹細胞および白血病幹細胞ではCD47を発現することによりマクロファージの貪食から逃れているが，抗CD47抗体処理することにより，マクロファージからの貪食が促進され，白血病幹細胞の除去につながる[9][10]．しかしながら，CD47ノックアウトマウスではEAE様脳炎をきたすことが知られており，CD47-SIRPαシグナル抑制が予期せぬ自己免疫をきたす危険性は残る．他に大腸がん幹細胞に発現するDLL4やIGF1Rを標的とした抗体の有用性が示唆されている[11][12]．

2）がん幹細胞と免疫逃避

がん幹細胞が免疫逃避を備えるという報告がなされている．Heimbergerらのグループは，ヒトグリオーマ組織およびグリオーマ細胞株から樹立されたグリオーマ起始細胞は免疫抑制性の細胞表面分子（B7-H1）および液性因子（Galectin-3，TGF-β1，VEGF，PGE_2）の発現が高いことを報告している．これら免疫抑制因子により，グリオーマ起始細胞は，T細胞増殖抑制，アポトーシス誘導，制御性T細胞（Treg）の誘導を起こす．反面，グリオーマ起始細胞を分化させると免疫抑制力は解除されることから，グリオーマ起始細胞特異的な免疫逃避機構と考えられる[13]．

CTLによる*in vitro*選択をくり返すことにより樹立されたCTL耐性がん細胞亜株では，幹細胞マーカーの1つであるNanogの発現が高くなり，がん幹細胞形質を獲得するとともに，アポトーシス抑制分子であるMcl1発現を誘導することによりCTL耐性を獲得しているとの報告がある[14]．幹細胞分子が直接免疫逃避にかかわっている可能性を示唆する．

2 EMTを起こすがん細胞亜集団

EMTを起こし，間質に浸潤するがん細胞では間質を分解するエフェクター分子であるマトリックスメタロプロテアーゼ（MMP）群を発現する．MMPはCTLの標的となることが報告されている．つまり，EMTを起こしているがん細胞は新たな抗原性を獲得する．その反面，免疫細胞が豊富な，いわば免疫細胞のテリトリーである間質に新たな抗原性を獲得したがん細胞が浸潤するためには，免疫から逃避するためのメカニズムが備わっている．

1）EMTの抗原性

EMT形質を獲得したがん細胞は，さまざまな間質を分解する酵素群を発現し，間質に浸潤する．MMP-2は細胞接着分子であるインテグリンαvβ3依存性に悪性黒色腫細胞に提示されることが報告されている[15]．また，さまざまながん腫で発現するMMP-7もCTLの標的となることが報告されている[16]．EMTを起こしたがん細胞が間質に浸潤するうえで，重要な役割をもつMMPが免疫の標的にもなる．

2）EMTと免疫逃避

転写因子 Snailによって誘導されたEMTは，がん組織内への樹状細胞浸潤を抑制し，逆に免疫抑制性の制御性T細胞（Treg）浸潤を促進することにより，がんが免疫機構から逃避する[17]．また，Snail特異的なsiRNAにより，樹状細胞の浸潤が促進され，Treg浸潤は抑制される．

一方では，neuトランスジェニックマウスという人工的発がんモデルではあるが，同モデルではCTLが，発がんに深くかかわっているとの報告がある．CTLが分泌するエフェクターサイトカインの1つであるTNF-αががん細胞のEMTおよびがん幹細胞形質を有する$CD44^+CD24^-$細胞を誘導する[18]．他にも，TNF-αがNF-κBシグナルを介してTwist発現およびEMTを誘導することが知られている[19]．抗原ペプチドに対する免疫応答が起き，CTLが活性化するとTNF-αが分泌される．TNF-αは標的細胞にアポトーシスを誘導する一方で，標的細胞にEMTまたはがん幹細胞形質を誘導し，免疫逃避につながる可能性がある．免疫応

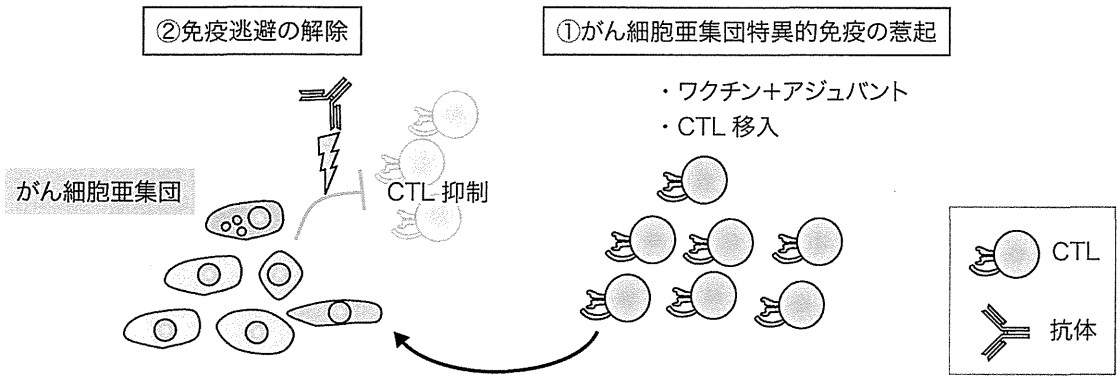

図3　がん細胞亜集団標的免疫療法
がん細胞亜集団に対する有効な免疫応答を誘導するためには，がん細胞亜集団特異的免疫応答の惹起（①）およびがん細胞亜集団による免疫逃避機構の解除（②）が必要と考えられる．①がん細胞亜集団特異的免疫を惹起するために，ワクチンにアジュバントを併用する，がん細胞亜集団特異的CTLの移入などが考えられる．②免疫逃避の解除にはさまざまなモノクローナル抗体が有効である可能性が示唆される

答のネガティブ・フィードバック機構があることを示唆する．

3 オートファジーを起こすがん細胞亜集団

飢餓状態に陥った細胞は，オートファジーという自食機構により，細胞生存に必要なアミノ酸を確保することが知られている．がん細胞は恒常的にオートファジー機構がオンになっていることが知られており，細胞のがん化およびがん細胞の維持に重要な働きをしている．がん細胞でオンになっているオートファジーにより，新たな抗原ペプチドが産生されることが知られており，がん細胞が新たな免疫原性を獲得することが知られている反面，オートファジーが免疫抑制を誘導することも知られている．

1）オートファジーの抗原性

がん細胞では，飢餓状態ではないにもかかわらずオートファジーが活性化していることが知られている．がん細胞で活性化するオートファジーにより，広範な正常細胞にも発現する分子であるPSA（puromycin-sensitive aminopeptidase）から新たな抗原ペプチドが産生されることが報告されている[20]．がんは，細胞増殖が早いため，相対的な血流不足のため壊死を起こすことが多い．特にこのような低栄養，低酸素状況の部位では，オートファジーが誘導され，新たな抗原ペプチドが産生される可能性が示唆される．

2）オートファジーと免疫逃避

Chouaibらは低酸素で誘導されるオートファジーが，CTL抵抗性を誘導することを示した[21]．低酸素により安定化するHIF1αが，Srcキナーゼ活性化を介してSTAT3を活性化する．低酸素で誘導されるオートファジーをBeclin-1特異的shRNAで抑制すると，免疫療法の効果が増強される．さらに，同グループは$CD44^+$ $CD24^-$ $ALDH1^+$の乳がん幹細胞は，EMTを起こしている細胞群であり，なおかつ，オートファジー依存性にCTL耐性を獲得していることを報告している．前記に述べてきたがん幹細胞形質，EMT形質およびオートファジーが互いに重なり合う形質であり，CTL耐性につながることを示している[22]．

おわりに

がんは，さまざまな免疫逃避機構を備えて発症する．がん幹細胞，EMTを起こす細胞，オートファジーを起こす細胞などのがん細胞亜集団をつくるうえでさらなる免疫逃避機構を獲得することが判明してきた．一方では，がん細胞亜集団形質を獲得すると同時に，必ず新たなタンパク質の発現が誘導される．同タンパク質は新規の抗原分子としてCTLなどの免疫エフェクターに認識される標的となりうる．つまり，がん細胞亜集団を標的とするには，新たな抗原分子に対する免疫応答の惹起に加え，免疫逃避機構の解除が必要になると考えられる（**図3**）．免疫応答の惹起には，ペプチドワクチンのみならず，アジュバントや，抗原ペプチド特異的CTL移入療法が有効かもしれない．また，免疫逃避機構の解除には，抗CTLA-4抗体，抗PD-1抗体

（**玉田の稿**参照），抗CCR4抗体などの抗体医薬が期待される．がん細胞母集団を標的とする免疫療法に加えて，より生物学的悪性度の高いがん細胞亜集団を漏らさず狙える併用免疫療法をデザインすることが，免疫療法を成功させるキーの1つと考えられる．

文献

1）Inoda, S. et al. : Am. J. Pathol., 178 : 1805-1813, 2011
2）Tallerico, R. et al. : J. Immunol., 190 : 2381-2390, 2013
3）Hirohashi, Y. et al. : Oncoimmunology, 1 : 123-125, 2012
4）Nishizawa, S. et al. : Cancer Res., 72 : 2844-2854, 2012
5）Boiko, A. D. et al. : Nature, 466 : 133-137, 2010
6）Yamada, R. et al. : Tissue Antigens, 81 : 428-434, 2013
7）Castriconi, R. et al. : J. Immunol., 182 : 3530-3539, 2009
8）Todaro, M. et al. : J. Immunol., 182 : 7287-7296, 2009
9）Jaiswal, S. et al. : Cell, 138 : 271-285, 2009
10）Majeti, R. et al. : Cell, 138 : 286-299, 2009
11）Hoey, T. et al. : Cell Stem Cell, 5 : 168-177, 2009
12）Dallas, N. A. et al. : Cancer Res., 69 : 1951-1957, 2009
13）Wei, J. et al. : Clin. Cancer Res., 16 : 461-473, 2010
14）Noh, K. H. et al. : J. Clin. Invest., 122 : 4077-4093, 2012
15）Godefroy, E. et al. : J. Exp. Med., 202 : 61-72, 2005
16）Yokoyama, Y. et al. : Clin. Cancer Res., 14 : 5503-5511, 2008
17）Kudo-Saito, C. et al. : Cancer Cell, 15 : 195-206, 2009
18）Santisteban, M. et al. : Cancer Res., 69 : 2887-2895, 2009
19）Li, C. W. et al. : Cancer Res., 72 : 1290-1300, 2012
20）Demachi-Okamura, A. et al. : PLoS One, 7 : e47126, 2012
21）Noman, M. Z. et al. : Cancer Res., 71 : 5976-5986, 2011
22）Akalay, I. et al. : Cancer Res., 73 : 2418-2427, 2013

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廣橋良彦：1996年，和歌山県立医科大学卒業後，2002年，同大学にて学位取得．大学院時代に札幌医科大学第一病理にて腫瘍免疫研究に従事．'02年，英国オックスフォード大学および米国ペンシルバニア大学 Mark Greene ラボに留学後，'05年日本学術振興会特別研究員を経て，'08年より札幌医科大学・助教（佐藤昇志教授）．本当に効くがん免疫療法を確立すべく，がんのバイオロジーを免疫学の側面から斬ることを心がけています．

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MINI-REVIEW

Equol, Adiponectin, Insulin Levels and Risk of Breast Cancer

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Abstract

Breast cancer is one of the most frequently diagnosed cancers and the leading cause of cancer death among women. Soy isoflavones have been widely studied and among all isoflavones equol has been gaining interest with regard to its relationship with breast cancer risk. Obesity has been revealed as one of the breast cancer risk factors, known to be associated with high levels of circulating insulin and decreased levels of adiponectin. Hence there have been many studies investigating relationships between insulin and adiponectin levels and breast cancer risk. Additionally recent findings have suggested that insulin and adiponectin themselves may have influence on breast cancer development, independent of obesity. In the present review, we discuss the relationships between breast cancer risk and equol, insulin and adiponectin levels, which are three important factors in our ongoing hospital-based case-control study. Herein these factors are reviewed not only from the clinical viewpoint but also from possible chemical and biological points of view which may explain clinical observations.

Keywords: Breast cancer - isoflavones - equol - insulin - adiponectin - menopausal status

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Introduction

Breast cancer is one of the most frequently diagnosed cancers and the leading cause of cancer death among women. There has been interest in protective role of soy isoflavones against breast cancer. Soy isoflavones are widely studied and the data have shown their biological activities such as an affinity for estrogen receptor, which may act as antiestrogens by competing for the binding sites of estrogen receptors (Martin et al., 1978). Among all the isoflavones, equol, a metabolite of daidzein, has gaining interest due to its possible effects on cancer risk. *In vitro* studies equol was found to be more biologically active than daidzein, with a higher affinity for the estrogen receptor and a more potent antioxidant activity, and this suggests that it may be advantageous to convert daidzein to equol to enhance its estrogenic potency. Some epidemiological studies suggest that the ability to produce equol is associated with reduced risk of breast cancer while others have reported no or adverse effects (Trock et al., 2006; Wu et al., 2008; Dong et al., 2011). One of the potential reasons for these inconsistences could be inter-individual differences in isoflavone metabolism (Setchell et al., 2002; Heinonen et al., 2003; Setchell and Cole, 2006). Most of these studies did not determine the equol producer status. According to studies approximately only 30-50% of individuals are capable to produce equol (Hutchins et al., 1995; Kelly et al., 1995; Lampe et al., 1998; Arai et al., 2000; Akaza et al., 2002). However, how one becomes an equol producer remains unknown. Recent studies suggest that soy isoflavones may provide a clinical benefit for breast cancer (Lampe, 2010) however, in most part the role of equol in relation to breast cancer remains unclear.

Additionally many studies have established that obesity is one of the common factors in breast cancer especially in postmenopausal women. Obesity is associated with high levels of circulating insulin. Insulin has shown to stimulate cell proliferation in normal breast tissue and in human breast cancer cell lines (Ish-Shalom et al., 1997; Chappell et al., 2001) and enhanced breast tumor growth in animal models (Shafie and Grantham, 1981; Shafie and Hilf, 1981). Some epidemiological studies have measured insulin to evaluate its association with breast cancer development. However, only few studies providing data on fasting levels of insulin and breast cancer found no consistent association.

Furthermore obesity and excessive adipose tissue is known to lead to decreased production of the peptide adiponectin. Adiponectin circulates in the plasma at concentrations correlates inversely with body mass index (BMI) (Vona-Davis and Rose, 2007). Thus it has been proposed that adiponectin may be a biological link between obesity and increased breast cancer risk. It is considered that adiponectin may influence on breast cancer risk through its effects on insulin resistance but some recent studies suggest that adipose tissue-derived

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hormones, including adiponectin may also be directly involved in breast cancer development (Miyoshi et al., 2003; Mantzorof et al., 2004; Petridou et al., 2004; Rose et al., 2004; Lorincz et al., 2006; Tworonger et al., 2007) and some studies revealed the antiangiogenic and antitumoral effects of adiponectin (Kumor et al., 2009). However, relatively small numbers of cohort studies have examined breast cancer risk in association with adiponectin levels and remain inconclusive.

As mentioned above obesity is considered to be one of the risk factors of breast cancer, there have been researches focused on other adipokines such as resistin (Kang et al., 2007) which is increased in obesity (Steppan et al., 2001) and leptin (Chen et al., 2006) which increase with BMI (Ruhl and Everhart, 2001) and on insulin resistance (Oh et al., 2011; Al Awadhi et al., 2012), which is linked to obesity as well (Rajala and Scherer, 2003). Additionally several studies have used C-peptide as a maker of insulin secretion instead of using direct measurements of insulin levels (Bruning et al., 1992; Yang et al., 2001) or used glucose levels which consequently increase as insulin resistance increases (Kaaks, 1996; Vona-Davis et al., 2007). Likewise, there have been studies focused not only on insulin levels but also on related factors such as insulin-like growth factor I (IGF-1), insulin-like growth factor binding proteins (IGFBPs), (Jernstrom and Barrett-Connor, 1999; Kaaks et al., 2002; Gunter et al., 2009).

In our ongoing hospital-based case-control study we focus particularly on the following three factors, equol, insulin and adiponectin levels in serum and investigate the relationships between breast cancer risk and these factors. Herein, we reviewed articles which discussed breast cancer risk in relation to equol, adiponectin and insulin, which are considered to be important as risk factors and biomarkers of breast cancer. To better understand the potential involvement of these factors in breast cancer, we examined not only clinical observations but also possible chemistry and biology behind these clinical observations.

Equol

Chemistry and biological property of equol

Equol is a chiral molecule and can exist as two isomers, R- and S-equol. S-(-)-equol is the metabolite of daidzein by intestinal bacteria. Equol is expected to prevent hormone-dependent diseases including breast cancer due to its ability to bind both estrogen receptors (ER) α and β (Kinjo et al., 2004; Muthyala et al., 2004), especially S-(-)-equol is known to have a much stronger affinity for ER β compared to R-(+)-equol (Setchell et al., 2005; Jackson et al., 2011), and moreover due to its superior anti-oxidative potential to all the isoflavones (Arora et al., 1998; Rufer et al., 2006). In addition, equol binds to sex hormone binding globulin and competitively inhibits estradiol and testosterone binding in a dose-dependent manner (Martin et al., 1996). Only little research that focused on equol specifically has conducted whereas mechanisms of soy isoflavone on breast cancer have been well done. *In vitro* studies have demonstrated that equol, both racemic and S-equol inhibited the growth of the breast cancer cell line MDA-MB-231 at higher concentration (≥10 μM) but in contrast, equol at lower concentration (≤10 μM) stimulated the proliferation of ER positive breast cancer cells. The compounds also showed effects in inhibiting the invasion of MDA-MB-231 cancer cells through matrigel (Magee et al., 2006). Another study reported that (±)-equol have proliferative effects on MCF-7 cell growth *in vitro* within the concentration of plasma equol 2.10-3.21 μM. *In vivo* study internal exposures to equol did not stimulate growth of estrogen-dependent human breast tumor (MCF-7) growth, increase the cell proliferation in tumors or induce an estrogen-responsive pS2 expression (Ju et al., 2006). It is difficult to interpret the effect of equol on breast cancer since findings from *in vitro* studies did not necessarily support *in vivo* studies. There is the need for more *in vivo* studies to explain *in vitro* results.

Epidemiological studies of equol on breast cancer

Few epidemiological studies have investigated the association between equol and breast cancer risk. Some results have suggested positive relationship between equol exposure and breast cancer risk. Ingram et al. (1997) conducted a case-control study to assess the association between phytoestrogen intake and the risk of breast cancer. Urine collection and blood samples from 144 pairs of cases and controls were analyzed. Their findings were that increasing excretion of equol was associated with a significant reduction in risk of breast cancer development. The risk for the highest quartile of excretion was one quarter that of the lowest quartile of excretion after the adjustment (odds ratio (OR), 0.27; 95% confidence interval (CI), 0.10-0.69; P=0.009), represented a four-fold reduction in risk. Menopausal status was not mentioned. In their conclusion, there was a substantial reduction in breast cancer risk among women with a high intake (as measured by excretion) of isoflavonoid phytoestrogen, equol. Furthermore, two ethnic based studies have reported. Goodman et al. (2009) examined the association of urinary phytoestrogens with the risk of postmenopausal breast cancer in the multiethnic cohort study. A nested case-control study of 251 cases and 462 controls was conducted in Hawaii. The risk of breast cancer was reduced among White women with the highest compared with the lowest quartile excretion of equol (OR, 0.27; 95%CI, 0.08-0.95), although the trend risk was not significant (P=0.07). No relation of urinary equol to the risk of breast cancer was found among all subjects. Their findings may support the hypothesis that diet rich in isoflavones from soy products reduced the risk of postmenopausal breast cancer, particularly in populations with comparatively high excretion of phytoestrogens. Similarly analyses of a case-control study of breast cancer conducted among Asian-American (Chinese, Filipino and Japanese) women ages of 25 and 74 years in Los Angeles County by Wu et al. (2004) showed 49% of controls had measurable levels of plasma equol compared with 39% of cases, but the difference was not statistically significant. A nested case-control study by Verheus et al. (2007) investigated the association between plasma isoflavone levels and breast cancer risk in a prospective manner in Dutch population. Plasma levels of including equol of 383 cases and 383 controls

were measured. Women with detectable equol levels were shown to have decreased breast cancer risk, compared with women with nondetectable levels (OR, 0.87; 95%CI, 0.63-1.21), and when women with detectable levels above the median were compared with women with nondetectable levels, the protection was somewhat stronger (OR, 0.77; 95%CI, 0.49-1.21). Results were the same in pre- or perimenopausal women and in postmenopausal women. In their conclusion, there was no statistically significant association with reduced breast cancer risk and plasma equol. Zheng et al. (1999) conducted a population-based case-control study in the urban Shanghai area to evaluate urinary excretion of isoflavonoids and risk of breast cancer. 60 case-control pairs who were selected and their urine samples were assayed for isoflavonoids. In their result, mean urinary equol was lower in cases than controls, however, OR and 95%CI for the association of breast cancer with urinary excretion of equol was not analyzed because of the difficulty in making groups of cases and controls due to its extremely low levels in urine samples. In their summary, despite a small sample size, this study showed that urinary excretion of total isoflavonoids was substantially and statistically significantly lower in breast cancer patients than in controls in a population with generally high soy consumption, however, did not show specifically in equol excretion. In contrast, Grace et al. (2004) investigated phytoestrogen concentrations of women aged 45-75 years in serum and urine as biomarkers for dietary phytoestrogen intake and their relation to breast cancer risk in UK. Measurements of 114 spot urines and 97 serum samples from breast cancer cases were compared with those of 219 urines and 187 serum samples from healthy controls. They have found that for urine, 39% of cases were equol producers compared with 31% of controls and for serum, 39% of cases were equol producers compared with 37% of controls. In this study menopausal status was not stated. In their conclusion, exposure to all isoflavones was associated with increased breast cancer risk, significantly so for equol. For a doubling of levels, OR increased by 20-45% (OR, 1.34; 95%CI, 1.06-1.70, P=0.013 for urine equol and OR, 1.46; 95%CI, 1.05-2.02, P=0.024 for serum equol). In the follow up study, Ward et al. (2008) analyzed phytoestrogens in serum and urine samples of breast cancers cases and control individuals in the European Prospective into Cancer-Norfolk (aged 45-75 years). Although their results showed that breast cancer risk was marginally increased with higher levels of total urinary isoflavones; among those with estrogen receptor-positive tumors, the risk of breast cancer was increased with higher levels of urinary equol (OR, 1.07; 95%CI, 1.01-1.12, P=0.013), in their conclusion, no association between serum or urinary equol and breast cancer risk was found. Among seven articles reviewed, only one result showed that equol exposure increased breast cancer risk, while three studies observed no association between equol exposure and breast cancer risk, remaining articles showed reduced breast cancer risk with higher equol measurements but with limitations. These inconsistent findings from epidemiological studies can be explained by the fact that these studies have been conducted in Western populations who generally have the low isoflavone intake and Asian populations who tend to consume high amount of isoflavones. Isoflavone intake in Europe and USA is about 3 mg/d (Messina, 2010) whereas in Japan and Chinese cities is 25-50 mg/d (Ju et al., 2006). Differences from these results suggest that isoflavone metabolism may differ between countries and/or ethnic groups due to differences in intestinal microflora. Furthermore some of these epidemiological studies stated their limitations in relatively small number of samples, which may be a cause of inconsistency in results. Other effect modifiers including ER/PR status, pre/postmenopausal status and equol producing status are also needed to be considered when analyzing epidemiological study results comprehensively.

Insulin

Chemistry and biological property of insulin

The primary translation product of the insulin gene is preproinsulin, a 110-amino-acid-long peptide that is processed in the pancreatic β-cells to yield proinsulin. Risk of breast cancer is increased in association with obesity, which is characterized by increased insulin resistance, with consequent increase in circulating levels of insulin. Insulin is most widely known for its metabolic effects (Scheen, 1996; Whitelaw, 1998), but it has mitogenic effects as stimulating cell mitosis and migration and inhibiting apoptosis in DNA damaged untransformed breast epithelial cells as well (Merlo et al., 1995). Generally the metabolic effects of insulin are mediated by way of the phosphatidylinositol 3-kinase (PI3K) pathway, while the mitogenic effects of insulin are the activation of Ras and the mitogen-activated protein kinase (MAPK) pathway (Rose and Vona-Davis, 2012). The capacity for stimulation of the PI3K pathway by insulin is lost when insulin resistance with hyperlipidemia is present, but MAPK activation is enhanced and insulin-induced prenylation of Ras protein is increased (Gallagher and LeRoith, 2010; Drazin, 2011). Insulin may also increase the risk of breast cancer by alterations in circulating estrogen levels. Chronic hyperlipidemia is associated with increased ovarian estrogen production, reduced hepatic secretion of sex hormone-binding globulin, and increased free estradiol levels (Porestsky et al., 1987; Pugeat et al., 1991, Calle and Kaaks 2004). Insulin is also known to promote cell proliferation in normal breast tissue and in human breast cancer cell lines (Ish-Shalom et al., 1997; Chappell et al., 2001) and enhance breast tumor growth in animal models (Shafie and Grantham, 1981). A number of cell culture experiments have been performed to examine the effect of insulin on breast cancer cell growth. The growth of the ER-(+) MCF-7, T47D and ZR-75-1 human breast cancer cell lines were promoted by insulin addition, and in all cases, insulin stimulated insulin receptor (IR) tyrosine kinase activity and thymidine incorporation (Ogasawara and Sirbasku, 1988; Milazzo et al., 1992). The effects of insulin on ER-(-) human breast cancer cell lines were complicated and the interpretation was limited. Gliozzo et al. (1998) reported that cultured MDA-MB-157 cells showed a strong mitogenic response to exogenous insulin. Malaguarnera et al. (2012) also used the same cell line and

observed similar stimulatory effects on breast cancer cell proliferation. In contrast, two other ER-(-) human breast cancer cell lines, MDA-MB-468 and MDA-MB-231, did not show a significant mitogenic response (Osborne et al., 1978; Sepp-Lorenzino et al., 1994; Belfiore et al., 1996).

Epidemiological studies of insulin on breast cancer

To our knowledge, there are only a few prospective studies have directly assessed the association between insulin levels and breast cancer risk. Del Giudice et al. (1998) conducted a case-control study comparing plasma insulin levels in 99 premenopausal women with breast cancer and 99 controls to proof the hypothesis that insulin may play an important role in development of breast cancer. They found that elevated insulin levels were significantly associated with breast cancer after adjusting age and weight and the risk was higher in the highest insulin quintile compared to the lowest quintile (OR, 2.83; 95%CI, 1.22-6.58). The result was independent of diet and other known risk factors for breast cancer. In their conclusion, circulating insulin levels are elevated in women with premenopausal breast cancer. In contrast to the previous report, the other report from a case-control study of investigating the relationship between breast cancer and fasting insulin (Jernstrom, 1999) revealed that fasting insulin was significantly positively correlated with both current weight and weight gain but the levels of insulin did not differ significantly between women with and without breast cancer. They concluded that the increased risk of breast cancer was not associated with fasting insulin. Kaaks et al. (2002) conducted measurements of plasma concentration of insulin within two prospective cohorts of 513 breast cancer cases and 987 controls. They found that breast cancer risk showed no clear associations with levels of insulin and this result did not support the hypothesis that elevated plasma insulin levels were associated with increased breast cancer risk, which was supporting Jernstorm results. Mink et al. (2002) examined the association of breast cancer incidence with serum levels of insulin in a cohort of 7894 women aged 45-64 years from four US communities. 187 breast cancer cases were ascertained after average 7.1 years of fellow-up period. They found that breast cancer was positively associated with BMI but not with serum insulin level and concluded that circulating insulin levels were not predictable of future breast cancer incidence. Muti et al. (2002) analyzed the hypothesis that serum insulin levels were associated with breast cancer using a nested case-control study in Italy. 133 breast cancer cases and 503 controls were finally analyzed and in their results, insulin showed a weaker association with breast cancer, the adjusted relative risk of the highest quartile vs. the lowest was 1.7 (95%CI, 0.7-4.1, P trend=0.14) in premenopausal women, but not in postmenopausal women. They also found that both pre- and postmenopausal women, insulin was positively related to BMI (r=0.30 for both groups, P<0.005). Gunter et al. (2009) conducted a case-cohort study of incident breast cancer among postmenopausal women. They observed a strong positive association between the risk of breast cancer and fasting insulin levels in postmenopausal women with hazard ratio (HR) for highest vs. lowest quartile of insulin level was 1.46 (95%CI, 1.00-2.13; P trend, 0.02), however, the association with insulin level varied by hormone therapy (HT) use. The association was observed only in women with non HT users with HR for highest vs. lowest quartile of insulin level was 2.40 (95%CI, 1.30-4.41; P trend<0.001) after the adjustments, and this finding was consistent with data from other studies that showed that HT use interacts with the association between obesity and postmenopausal breast cancer (Lahmann et al., 2004; Li et al., 2006; Ahn et al., 2007). In their summary, hyperlipidemia was an independent risk factor for postmenopausal breast cancer and therefore interventions aimed at lowering fasting insulin levels may reduce the risk of breast cancer in postmenopausal women. Kabat et al. (2009) conducted a longitudinal study of postmenopausal breast cancer risk. They reported that mean insulin levels were higher in cases compared to non-cases at baseline and at years 1 and 3 with statistically non-significant difference whereas in year 6 mean insulin was lower in cases compared to non-cases. They also found that baseline insulin levels were positively associated with breast cancer risk with statistically significant linear trends over increasing tertiles. For all participants, the multivariable hazard ratio for the highest tertile of serum insulin compared to the lowest was 2.22 (95%CI, 1.39-3.53). Additionally the association of insulin with breast cancer was strongest among lean women (BMI<25 kg/m^2) and weakest among obese women (BMI ≥ 30 kg/m^2). They confirmed that the association of insulin with breast cancer was significant regardless of hormone use status. Their results suggest that baseline insulin levels were positively associated with risk of postmenopausal breast cancer. Among seven studies reviewed in this paper, three articles stated that there are significant associations between insulin levels and breast cancer, one article observed the same only in premenopausal women, while three articles concluded that there are no significant associations. Given these inconsistent considerations, further investigations of effects of insulin on breast cancer are warranted.

Adiponectin

Chemistry and biological property of adiponectin

Adiponectin is a 224-amino-acid-long polypeptide hormone and known as one of the adipokines. Adiponectin, the gene product of the adipose most abundant gene transcript 1 (apM1) and adiponectin cDNA was first isolated by large scale random sequencing of the human adipose tissue cDNA library. It is a collagen-like protein that is exclusively synthesized in white adipose tissue, is induced during adipocyte differentiation, and circulates at relatively high concentrations in the serum (Maeda et al., 1996). Its effects on the metabolic process such as gluconeogenesis, glucose uptake, lipid-β-oxidation, triglyceride clearance, protection from endothelial dysfunction, insulin sensitivity and weight loss have been revealed (Arita et al., 1999; Matsuzawa et al., 1999; Weyer et al., 2001; Okamoto et al., 2002; Yamauchi et al., 2003). Adiponectin level was found to be inversely correlated to body fat percentage and it tend to be lower

in obesity (Arita, 1999). It is also known that levels of adiponectin are reduced in diabetics compared to non-diabetics. Besides these metabolic effects, adiponectin has been shown to suppress proliferation of macrophages (Yokota et al., 2000, Diez, 2003), to lead endothelial cell apoptosis and reduction of tumor vascularization by inducing caspase enzyme activation (Brakenhielm et al., 2004). Furthermore, it has been reported that MCF-7 breast cancer cells responded to adiponectin by reducing their growth, AMP kinase activation and p42/p44 MAP kinase inactivation receptors (Dieudonne et al., 2006) and others also reported that adiponectin can inhibit proliferation of MCF-7 breast cancer cells (Arditi et al., 2007; Jarde et al., 2008). Korner et al. (2007) have reported that exposure of T47D breast cancer cells to adiponectin significantly inhibited the percentage of viable cells and proliferation and therefore suggesting that adiponectin may act by not only altering the hormonal milieu but also directly inhibiting the proliferation of breast cancer cells *in vitro*. Also it was reported that adiponectin can suppress cell growth in ER-(-) MDA-MB-231 breast cancer cell line (Kang et al., 2005; Wang et al., 2006). Despite these experimental results, the mechanisms underlying the proliferation effects of adiponectin are not fully understood. Studies of the effects of adiponectin on the apoptosis of breast cancer cells *in vitro* lead inconclusive results, some reported that the apoptosis was increased by adiponectin treatment but others did not observe the same results.

Epidemiological studies of adiponectin on breast cancer

While several studies have suggested conflicting and controversial role for adiponectin in breast cancer risk, some research have reported associations between adiponectin levels and breast cancer risk from epidemiological aspect. Miyoshi et al. (2003) conducted a case-control study on 102 breast cancer patients and 100 healthy women to examine the association of the serum adiponectin levels with breast cancer risk. They have found that women in the low tertile of serum adiponectin levels were associated with a significantly ($P<0.05$) increased risk for breast cancer compared with women in high tertile (OR, 3.63; 95%CI, 1.61-8.19). This association was observed both in the premenopausal women (OR, 3.45, 95%CI, 0.89-13.50) and in the postmenopausal women (OR, 3.90; 95%CI, 1.23-12.44). Besides the frequency of large (>2cm) tumors and that of high histological grade (2+3) tumors were significantly higher in breast cancer patients in the low tertile of the serum adiponectin levels than those in the high and intermediate tertiles ($P<0.005$ and $P<0.05$, respectively). In their conclusion, the low serum adiponectin levels are significantly associated with an increased risk for breast cancer and that tumors arising in women with the low serum adiponectin levels are more likely to be biologically aggressive phenotype. Mantzoros et al. (2004) evaluated the association of adiponectin with the occurrence of breast cancer in a case-control study comprising 174 women with breast cancer and 167 controls. They have found an inverse, fairly strong, and statistically significant association of serum adiponectin with breast cancer (OR, 0.84; 95%CI, 0.71-0.99). This was only significant in postmenopausal women (OR, 0.82; 95%CI, 0.67-1.00) but not in premenopausal women. Chen et al. (2006) analyzed the correlations between the serum levels of adiponectin and the clinicopathological parameters in 100 breast cancer patients and 100 controls. They found serum levels were decreased significantly for adiponectin in the breast cancer patients in comparison to controls. ($P=0.003$). Among the clinicopathological parameters, ER, PR, HER2/neu, lymph node metastasis, tumor stage, and tumor grade all showed no effect on the serum levels of adiponectin. BMI was negatively correlated to serum adiponectin. In their conclusion, the results suggest that low serum adiponectin levels are associated with an increased risk for breast cancer. Kang et al. (2007) evaluated the relationship between serum adiponectin and breast cancer risk in 41 breast cancer patients and 43 controls. In their report the mean serum adiponectin levels was lower in the breast cancer group than the control group (6.93±3.2 μg/mL, 7.60±3.5 μg/mL, respectively), but this difference did not reach statistical significance ($P=0.37$). Besides there was no significant difference in serum adiponectin levels between cases and controls in either pre- or post-menopausal women ($P=0.22$, $P=0.89$, respectively). They also have investigated the relationship between serum adiponectin levels and clinicopathological characteristics of tumor. No significant difference was found in the frequency of large sized or highly differentiated tumors, or status of PR. The negativity of ER was significantly increased in the patients with less than the median adiponectin level ($P=0.032$). In their conclusion, the low serum adiponectin levels are likely to be associated with increased breast cancer risk in Korean women. Tworoger et al. (2007) conducted a prospective case-control study within the Nurses' Health Study (NHS) and NHSII to examine the association between plasma adiponectin concentrations and breast cancer risk. To our knowledge this is the largest case-control study of adiponectin levels and breast cancer risk up to the date including 1477 breast cancer cases and 2198 controls. They found that overall no association between plasma adiponectin levels and breast cancer risk, but there was a nearly significant interaction by menopausal status with a relative risk, top vs. bottom quartile of 0.73 (95%CI, 0.55-0.98; P trend=0.08) among postmenopausal women and 1.30 (95%CI, 0.80-2.10; P trend=0.09). Their results suggested that adiponectin may have an inverse association with breast cancer risk among postmenopausal women, particularly in a low-estrogen environment but have little or no association among premenopausal women. Korner et al. (2007) measured total and high-molecular weight (HMW) adiponectin in a hospital based case-control study of 74 breast cancer patients and 76 controls. In their results, women with the highest adiponectin levels had a 65% reduced risk of breast cancer ($P=0.04$). Using HMW instead of total adiponectin showed similar results. In addition to the *in vivo* study, they investigated the effect of adiponectin on proliferation in the T47D breast cancer cell line *in vitro* and found that the exposure of T47D cells to adiponectin significantly inhibited the percentage of viable cells to 86% and proliferation to 66% but had no

effect on apoptosis. They concluded that adiponectin may act as a biomarker of carcinogenesis. Tian et al. (2007) evaluated the association of measures of adiponectin with the development of breast cancer in a case-control study with 244 cases and 244 controls in Taipei. In their results, a fairly robust inverse association of adiponectin with the breast cancer risk was observed only in postmenopausal women (OR, 0.55; 95%CI, 0.23-0.97), but not in premenopausal women. Additionally, the plasma adiponectin levels tended to be inversely associated with ER-positive (OR, 0.53; 95%CI, 0.27-0.98), but not ER-negative breast tumors. In their conclusion, adiponectin may have an independent role in breast cancer carcinogenesis, particularly in the postmenopausal and ER-positive breast cancer risk. Oh et al. (2011) examined associations between breast cancer recurrence and adiponectin in a cohort of 747 patients. An inverse trend across the quartiles was observed for the serum adiponectin concentration in ER/PR-negative patients (P trend=0.027) but not in ER/PR-positive patients. Compared to the highest quartile for adiponectin level, the lowest quartile showed a hazard ratio of 2.82. Their findings suggested that assessing adiponectin concentrations may assist in establishing prognosis in ER/PR-negative cancers and interventions to increase serum adiponectin levels may represent a therapeutic option for reducing recurrence risk and improving prognosis in ER/PR-negative breast cancer. Gulcelik et al. (2012) evaluated the serum adiponectin levels in 87 breast cancer patients assessed the relation with menopausal status, receptor status and stage of disease. They have found that the serum adiponectin levels of breast cancer patients were lower than controls (8583±2095 ng/mL for cases and 13905±3263 ng/mL for control) and this difference was statistically significant (P<0.001). Also they found that the adiponectin levels decreased in relation to stage increases for breast cancer. Additionally there was no significant difference in adiponectin concentrations between pre- and postmenopausal breast cancer patients and adiponectin levels were not statistically different according to receptor status. In their conclusion, the low serum adiponectin level might be associated with breast cancer regardless of the menopausal and receptor status. Al Awadhi et al. (2012) evaluated the associations between circulating adipokines and breast cancer with 144 breast cancer cases and 77 controls. In their result, adiponectin level was significantly higher (P<0.05) in patients compared to controls. Their analysis showed that high levels of adiponectin (OR, 5.1; 95%CI, 2.2-11.5) was associated with breast cancer. In their conclusion, findings from their study confirmed that adipokines were associated with breast cancer. Among ten articles reviewed, three articles observed an inverse association between adiponectin levels and breast cancer risk only in postmenopausal women, one article observed the same result but only in ER/PR-negative breast cancer group. None of the articles showed an opposite observations in association with adiponectin levels and breast cancer risk. These accumulated clinical data support a role of adiponectin concentrations in breast cancer, however, to fully elucidate the mechanisms underlying the effect of adiponectin, larger prospective studies are needed.

Conclusion

In summary, a number of clinical and experimental data to suggest the importance of understanding effects of soy isoflavones, especially equol, insulin and adiponectin respectively on breast cancer. The evidence suggests that being an equol producer has a clinical benefit in some people, on the other hand, suggestions that isoflavones may have adverse effects in women with breast cancer or at increased risk of the disease has arisen mainly from animal studies and is generally not supported by clinical and epidemiological studies. Therefore interpretation of these results needs to be carried out carefully with consideration of equol producing status. Insulin action and endocrine and paracrine activities of the adipokines were established *in vitro* experiment, however, translation of its significance into human breast cancer needs to be conducted together with clinical and epidemiological studies. A role of adiponectin as a risk factor and possible diagnostic marker for breast cancer has been proposed, but again, biochemical mechanisms of how adiponectin influences on inhibiting breast cancer cell proliferation and leading apoptosis of breast cancer cell line are unclear. To elucidate the mechanism, additional studies of the expression and activation of adiponectin *in vitro* along with animal studies *in vivo* and clinical studies are essential. It is also very crucial to consider that the relationship between these factors; equol, insulin and adiponectin and breast cancer risk can differ depending on hormone receptor status and/or menopausal status. Hence further clinical and epidemiological analyses should be conducted including equol producing status, hormone receptor status and menopausal status for better understanding the precise role of these factors in breast cancer.

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References

Ahn J, Schatzkin A, Lacey JV Jr, et al (2007). Adiposity, adult weight change, and posymenopausal breast cancer risk. *Arch Intern Med*, **167**, 2091-102.

Akaza H, Miyanaga N, Takashima N, et al (2002). Is daidzein non-metabolizer a high risk for prostate cancer? A case-controlled study of serum soybean isoflavone concentration. *Jpn J Clin Oncol*, **32**, 296-300,

Al Awadhi SA, Al Khaldi RM, AL Rammah T, Kapila K, Mojiminiyi OA (2012). Associations of adipokines and insulin resistance with sex steroids in patients with breast cancer. *Indian J Med Res*, **135**, 500-5.

Arai Y, Uehara M, Sato Y, et al (2000). Comparison of isoflavones among dietary intake, plasma concentration and urinary excretion for accurate estimation of phytoestrogen intake. *J Epidemiol*, **10**, 127-35.

Arditi JD, Venihaki M, Karalis KP, Chrous GP (2007), Antiproliferative effect of adiponectin on MCF7 breast cancer cells: a potential hormonal like between obesity and cancer. *Horm Metab Res*, **39**, 9-13.

Arita Y, Kihara S, Ouchi N, et al (1999). Paradoxical decrease of an adipose specific protein, adiponectin in obesity. *Biochem Biophys Res Commun*, **257**, 79-83.

Arora A, Nair MG, Strasburg GM (1998). Antioxidant activities of isoflavones and their biological metabolites in a liposomal system. *Arch Biochem Biophys*, **356**, 133-41.

Belfiore A, Costantino A, Frasca F, et al (1996). Overexpression of membrane glycoprotein PC-1 in MDA-MB-231 breast cancer cells in associated with inhibition of insulin receptor tyrosine kinase activity. *Mol Endocrinol*, **10**, 1318-26.

Brackenheim E, Veitonmaki N, Cao R, et al (2004). Adiponectin-induced antiangiogenesis and antitumor activity involve caspase-mediated endothelial cell apoptosis. *Proc Natl Acad Sci USA*, **101**, 2476-81.

Bruning PF, Bonfrer JM, Van Noord PA, et al (1992). Insulin resistance and breast cancer risk. *Int J Cancer*, **52**, 511-6.

Calle EE, Kaaks R (2004). Overweight, obesity and cancer: epidemiological evidence and proposed mechanisms. *Nat Rev Cancer*, **4**, 579-91.

Chappell J, Leitner JW, Solomon S, et al (2001). Effect of insulin on cell cycle progression in MCF-7 breast cancer cells. Direct and potentiating influence. *J Biol Chem*, **276**, 38023-8.

Chen DA, Chung YF, Yeh YT, et al (2006). Serum adiponectin and leptin levels in Taiwanese breast cancer patients. *Cancer Lett*, **237**, 109-14.

Del Giudice ME, Fantus IG, Ezzat S, et al (1998). Insulin and related factors in premenopausal cancer risk. *Breast Cancer Res Treat*, **47**, 111-20.

Dieudonne MN, Bussiere M, Dos Santos E, et al (2006). Adiponectin mediates antiproliferative and apoptotic responses in human MCF7 breast cancer cells. *Biochem Biophys Res Commun*, **345**, 271-9.

Diez JJ, Iglesias P (2003). The role of the novel adipocyte-derived hormone adiponectin in human disease. *Eur J Endocrinol*, **148**, 293-300.

Dong JY, Qin LQ (2011). Soy isoflavones consumption and risk of breast cancer incidence or recurrence: a meta-analysis of prospective studies. *Breast Cancer Res Treat*, **125**, 315-23.

Drazin B (2011). Mechanism of the mitogenic influence of hyperinsulinemia. *Diabetology, Metabolic Syndrome*, **13**, 10.

Gallagher EJ, LeRoith (2010). The proliferating role of insulin and insulin-like growth factors in cancer. *Trends in Endocrinology and Metab*, **21**, 610-8.

Gliozzo B, Sung CK, Scala P, et al (1998). Insulin-stimulated cell growth in insulin receptor substrate-1-deficient ZR-75-1 cells in mediated by a phosphatidylinositol-3-kinase- independent pathway. *J Cellular Biochem*, **70**, 268-80.

Goodman MT, Shvetsov YB, Wilkens LR, et al (2009). Urinary phytoestrogen excretion and postmenopausal breast cancer risk: the multiethic cohort study. *Cancer Prev Res*, **2**, 887-94.

Grace PB, Taylor JI, Low YL, et al (2004). Phytoestrogen concentrations in serum and spot urine as biomarkers for dietary phytoestrogen intake and their relation to breast cancer risk in European prospective investigation of cancer and nutrition-norfolk. *Cancer Epidemiol Biomark Prev*, **3**, 698-708.

Gulcelik MA, Colakoglu K, Dincer H, et al (2012). Associations between adiponectin and two different cancers: breast and colon. *Asian Pac J Cancer Prev*, **13**, 395-8.

Gunter MJ, Hoover DR, Yu H, et al (2009). Insulin, insulin-like growth factor-I, and risk of breast cancer in postmenopausal women. *J Natl Cancer Inst*, **101**, 48-60.

Heinonen MS, Hoikkala A, Wahala K, Adlercreutz H (2003). Metabolism of the soy isoflavones daidzein, genistein and glycitein in human subjects. Identification of new metabolites having an intact isoflavonoid skeleton. *J Steroid Biochem Mol Bio*, **87**, 285-99.

Hutchins AM, Slavin JL, Lampe JW (1995). Urinary isoflavonoid phytoestrogen and lignin excretion after consumption of fermented and unfermented soy products. *J Am Diet Assoc*, **95**, 545-51.

Ingram D, Sanders K, Kolybaba M, Lopez D (1997). Case-control study of phyto-oestrogens and breast cancer. *Lancet*, **350**, 990-4.

Ish-Shalom D, Christoffersen CT, Vorwerk P, et al (1997). Mitogenic properties of insulin and insulin analogues mediated by the insulin receptor. *Diabetologia*, **40**, 25-31.

Jackson LR, Greiwe JS, Schwen RJ (2011). Emerging evidence of the health benefits of S-equol, an estrogen receptor β agonist. *Nutrition Reviews*, **69**, 432-48.

Jarde T, Caldefie-Chezet F, Damez M, et al (2008). Adiponectin and leptin expression in primary ductal breast cancer and in adjacent healthy epithelial and myoepithelial tissue. *Histopathology*, **53**, 484-7.

Jernstrom H, Barrett-Connor E (1999). Obesity, weight change, fasting insulin, proinsulin, C-peptide, and insulin-like growth factor-1 levels in women with and without breast cancer: the rancho bernardo study. *J Wom Health Gend Bas Med*, **8**, 1265-72.

Ju YH, Fultz J, Allred KF, Doerge DR, Helferich WG (2006). Effects of dietary daidzein and its metabolite, equol, at physiological concentrations on the growth of estrogen-dependent human breast cancer (MCF-7) tumors implanted in ovariectomized athymic mice. *Carcinogenesis*, **27**, 856-63.

Ju YH, Fultz J, Allred KF, et al (2006). Estimated Asian adult soy protein and isoflavone intakes. *Nutr Cancer*, **55**, 1-12.

Kaaks R (1996). Nutrition, hormons, and breast cancer: is insulin the missing link? *Cancer Causes Control*, **7**, 605-25.

Kaaks R, Lundin E, Manjer J, et al (2002). Prospective study of IGF-1, IGF-binding proteins, and breast cancer risk, in Northern and Southern Sweden. *Cancer Causes and Control*, **13**, 307-16.

Kabat GC, Kim M, Caan BJ, et al (2009). Repeated measures of serum glucose and insulin in relation to postmenopausal breast cancer. *Int J Cancer*, **125**, 2704-10.

Kang JH, Lee YY, Yu BY, et al (2005). Adiponectin induces growth arrest and apoptosis of MDA-MB-231 breast cancer cell. *Arch Pharm Res*, **28**, 1263-9.

Kang JH, Yu BY, Youn DS, (2007). Relationship of serum adiponectin and resistin levels with breast cancer risk. *J Korean Med Sci*, **22**, 117-21.

Kelly GE, Joannou GE, Reeder AY, Nelson C, Waring MA (1995). The variable metabolic response to dietary isoflavones in humans. *Proc Soc Exp Biol Med*, **208**, 40-3.

Kinjo J, Tsuchihashi R, Morito K, et al (2004). Interactions of phytoestrogens with estrogen receptors alpha and beta (III). Estrogenic activities of soy isoflavone aglycones and their metabolites isolated from human urine. *Biol Pharm Bull*, **27**, 185-8.

Korner A, Pazaitou-Panayioyou K, Kelesidis I, et al (2007). Total and high-molecular-weight adiponectin in breast cancer: in vitro and in vivo studies. *J Clin Endocrinol Metab*, **92**, 1041-8.

Kumor A, Daniel P, Pietruczuk M, et al (2009). Serum leptin, adiponectin, and resistin concentration in colorectal adenoma and carcinoma (CC) patient. *Int J Colorectal Dis*, **24**, 275-8.

Lahmann PH, Hoffmann K, Allen N, et al (2004). Body size and breast cancer risk: findings from the European Prospective Investigation into Cancer and Nutrition (EPIC). *Int J Cancer*, **111**, 762-71.

Lampe JW, Karr SC, Hutchins AM, Slavin JL (1998). Urinary equol excretion with a soy challenge: influence of habitual diet. *Proc Soc Exp Biol Med*, **217**, 335-9.

Lampe JW (2010). Emerging research on equol and cancer. *J Nutr*, **140**, 1369S-72S.

Li CI, Malone KE, Daling JR (2006). Interactions between body mass index and hormone therapy and postmenopausal breast cancer risk (United States). *Cancer Causes Control*, **17**, 695-703.

Lorincz AM, Sukumar S (2006). Molecular links between obesity and breast cancer. *Endocr Relat Cancer*, **13**, 279-92.

Maeda K, Okubo K, Shimomura I, et al (1996). cDNA cloning and expression of a novel adipose specific collagen-like factor, apM1 (adipose most abundant gene transcript 1). *Biochem Biophys Res Commun*, **221**, 286-9.

Magee PJ, Raschke M, Steiner C, et al (2006) Equol: acomparison of the effects of the racemic compound with that of the purified S-enantiomer on the growth, invasion, and DNA integrity of breast and prostate cells *in vitro*. *Nutr* Cancer, **54**, 232-42.

Malaguarnera R, Sacco A, Voci C, et al (2012). Proinsulin binds with high affinity the insulin receptor isoform A and predominantly activates the mitogenic pathway. *Endocrinol*, **153**, 2152-63.

Mantzoros C, Petridou E, Dessypris N, et al (2004). Adiponectin and breast cancer risk. *J Clin Endocrinol Metab*, **89**, 1102-7.

Martin PM, Horwithz KB, Ryan DS, McGuire WL, (1987). Phytoestrogen interaction with estrogen receptors in human breast cancer cells. *Endocrinol*, **103**, 1860-7.

Martin M, Haourigui M, Pelissero C, et al (1996). Interactions between phytoestrogens and human sex steroid binding protein. *Life Sci*, **58**, 429-36.

Matsuzawa Y, Funahashi T, Nakamura T (1999). Molecular mechanism of metabolic syndrome X: contribution of adipocytokines adipocyte derived bioactive substances. *Ann NY Acad Sci*, **892**, 146-54.

Merlo GR, Basolo F, Fiore L, Duboc L, Hynes NE (1995). p53-dependent and p-53- independent activation of apoptosis in mammary epithelial cells reveals a survival function of EGF and insulin. *J Cell Bio*, **128**, 1185-96.

Messina M (2010). A brief historical overview of the past two decades of soy and isoflavone research. *J Nutr*, **140**, 1350-4.

Milazzo G, Giorgino F, Damante G, et al (1992). Insulin receptor expression and function in human breast cancer cell lines. *Cancer Res*, **52**, 3924-30.

Mink PJ, Shahar E, Rosamond WD, Alberg AJ, Folsom AR (2002). Serum insulin and glucose levels and breast cancer incidence: the atherosclerosis risk in communities study. *Am J Epidemiol*, **156**, 349-68.

Miyoshi Y, Funahashi T, Kihara S, et al (2003). Association of serum adiponectin levels with breast cancer risk. *Clin Cancer Res*, **9**, 5699-704.

Muthyala RS, Ju YH, Sheng S, et al (2004). Equol, a natural estrogenic metabolite from soy isoflavones: convenient preparation and resolution of R- and S-equols and their differing binding and biological activity through estrogen receptors alpha and beta. *Bioorg Med Chem*, **12**, 1559-67.

Muti P, Quattrin T, Grant BJB, et al (2002). Fasting glucose is a risk factor for breast cancer: a prospective study. *Cancer Epidemiol Biomark Prev*, **11**, 1361-8.

Ogasawara M, Sirbasku DA (1988). A new serum-free method of measuring growth factor activities for human breast cancer cells in culture. *In Vitro Cell Dev Biol*, **24**, 911-20.

Oh SW, Park CY, Lee ES, et al (2011). Adipokines, insulin resistance, metabolic syndrome, and breast cancer recurrence: a cohort study. *Breast Cancer Res*, **13**, R34.

Okamoto Y, Kihara S, Ouchi N, et al (2002). Adiponectin reduces atherosclerosis in adipoprotein E-deficient mice. *Circulation*, **106**, 2767-70.

Osborne CK, Monaco ME, Lippman ME, Kahn CR (1987). Correlation among insulin binding, degradation, and biological activity in human breast cancer cells in longterm tissue culture. *Cancer Res*, **38**, 94-102.

Petridou E, Papadiamantis Y, Markopoulos C, et al (2004). Leptin and insulin growth factor I in relation to breast cancer (Greece). *Cancer Causes Control*, **11**, 383-8.

Poretsky L, Kalin MF, (1987). The gonadotropic function of insulin. *Endocr Rev*, **8**, 132-41.

Pugeat M, Crave JC, Elmidani M, et al (1991). Pathophysiology of sex hormone binding globulin (SHBG): relation to insulin. *J Steroid Biochem Mol Biol*, **40**, 841-9.

Rajala M, Scherer P (2003). The adipocyte- At the crossroads of homeostasis, inflammation, and atherosclerosis. *Endocrinol*, **144**, 3765-73.

Rose DP, Komninou D, Stephenson GD (2004). Obesity, adipocytokines, and insulin resistance in breast cancer. *Obes Rev*, **5**, 153-65.

Rose DP, Vona-Davis L (2012). The cellular and molecular mechanisms by which insulin influences breast cancer risk and progression. *Endocrine-Related Cancer*, **19**, 225-41.

Rufer CE, Kulling SE (2006). Angtioxidant activity of isoflavones and their major metabolites using different in vitro assays. *J Agric Food Chem*, **54**, 2926-31.

Ruhl CE, Everhart JE (2001). Leptin concentrations in the United States: relations with demographic and anthropometric measures. *Am J Clin Nutr*, **74**, 295-301.

Scheen Al, Lefebvre PJ (1996). Insulin action in man. *Diadete Metab*, **22**, 105-10.

Sepp-Lorenzino L, Rosen N, Lebwohl DE (1994). Insulin and insulin-like growth factor signaling are defective in the MDA-MB-468 human breast cancer cell line. *Cell Growth Differ*, **5**, 1077-83.

Setchell KDR, Brown NM, Lydeking-Olsen E (2002). The clinical importance of the metabolite equol - a clue to the effectiveness of soy and its isoflavones. *J Nutr*, **132**, 3577-84.

Setchell KDR, Clerici C, Lephart E, et al (2005) S-equol, a potent ligand for estrogen receptor b, is the exclusive enantiomeric form of the soy isoflavone metabolite produced by human intestinal bacterial flora. *Am J Clin Nutr*, **81**, 1072-9.

Setchell KDR, Cole SJ (2006). Method of defining equol-producer status and its frequency among vegetarians. *J Nutr*, **136**, 2188-93.

Shafie SM, Hilf R (1981). Insulin receptor levels and magnitude of insulin-induced responses in 7,12-dimethylbenz(a) anthracene-induced mammary tumors in rats. *Cancer Res*, **41**, 826-9.

Shafie SM, Grantham FH (1981). Role of hormones in the growth and regression of human breast cancer cell (MCF-7) transplanted into athymic nude mice. *J Natl Cancer Inst*, **67**, 51-6.

Steppan CM, Bailey ST, Bhat S, et al (2001). The hormone resistin links obesity to diabetes. *Nature*, **409**, 307-12.

Tian YF, Chu CH, Wu MH, et al (2007). Anthropometric measures, plasma adiponectin, and breast cancer risk. *Endocrine-Related Cancer*, **14**, 669-77.

Trock BJ, Hilakivi-Clarke L, Clarke R (2006). Meta-analysis of soy intake and breast cancer risk. *J Natl Cancer Inst*, **98**, 459-71.

Tworoger SS, Eliassen AH, Kelesidis T, et al (2007). Plasma adiponectin concentrations and risk of incident breast cancer. *J Clin Endocrinol Metab*, **92**, 1510-6.

Verheus M, van Gils CH, Keinan-Boker L, et al (2007). Plasma phytoestrogens and subsequent breast cancer risk. *J Clin Oncol*, **25**, 648-55.

Vona-Davis L, Rose DP (2007). Adipokines as endocrine, paracrine, and autocrine factors in breast cancer risk and progression. *Endocrine-Related Cancer*, **14**, 189-206.

Vona-Davis L, Howard-McNatt M, Rose DP (2007). Adiposity, type 2 diabetes and the metabolic syndrome in breast cancer. *Obes Rev*, **8**, 395-408.

Wang Y, Lam JB, Lam KS, et al (2006). Adiponectin modulates the glycogen synthase kinase-3β/β-catenin signaling pathway and attenuates mammary tumorigenesis of MDA-MB-231 cells in nude mice. *Cancer Res*, **66**, 11462-70.

Ward H, Chapelais G, Kuhnle GG, et al (2008). European Prospective into Cancer-Norfolk cohort. Breast cancer risk in relation to urinary and serum biomarkers of phytoestrogen exposure in the European Prospective into Cancer-Norfolk cohort study. *Breast Cancer Res*, **10**, 32.

Weyer C, Funahashi T, Tanaka S, et al (2001). Hypoadiponectinemia in obesity and type 2 diabetes: close association with insulin resistance and hyperinsulinemia. *J Clin Endocrinol Metab*, **86**, 1930-5.

Whitelaw DC, Gilbey SG (1998). Insulin resistance. *Ann Clin Biochem*, **35**, 567-83.

Wu AH, Yu MC, Tseng CC, Twaddle NC, Doerge DR (2004). Plasma isoflavone levels versus self-reported soy isoflavone levels in Asian-American women in Los Angeles County. *Carcinogenesis*, **25**, 77-81.

Wu AH, Yu MC, Tseng CC, Pike MC (2008). Epidemiology of soy exposures and breast cancer risk. *Br J Cancer*, **98**, 9-14.

Yamauchi T, Kamon J, Ito Y, et al (2003). Cloning of adiponectin receptors that mediate antidiabetic metabolic effects, *Nature*, **423**, 762-9.

Yang G, Lu G, Jin F, et al (2001). Population-based case-control study of blood C-peptide level and breast cancer risk. *Cancer Epidemiol Biomark Prev*, **10**, 1207-11.

Yokota T, Oritani K, Takahashi I, et al (2000). Adiponectin, a new member of the family of soluble defense collagens, negatively regulates the growth of myelomonocytic progenitors and the functions of macrophages. *Blood*, **96**, 1723-32.

Zheng W, Dai Q, Custer LJ, et al (1999). Urinary excretion of isoflavonoids and the risk of breast cancer. *Cancer Epidemiol Biomark Prev*, **8**, 35-40.

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Human endoplasmic reticulum oxidoreductin 1-α is a novel predictor for poor prognosis of breast cancer

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Human endoplasmic reticulum oxidoreductin 1-α (hERO1-α) is an oxidizing enzyme that exists in the endoplasmic reticulum and its expression is augmented under hypoxia. It regulates a redox state of various kinds of protein through reoxidation of "client" protein disulfide isomerase. Interestingly, although the expression of hERO1-α in normal tissues was comparatively limited, various types of cancer cells expressed it in large amounts. Therefore, we examined the role of ERO1-α in tumor growth using murine breast cancer line 4T1 and found that knockdown of murine ERO1-α inhibited *in vivo* tumor growth and decreased lung metastasis compared with wild-type 4T1. Moreover, we investigated the relationship between expression of hERO1-α and prognosis in breast cancer patients. Seventy-one patients with breast cancer who underwent surgery between 2005 and 2006 in Sapporo Medical University Hospital (Sapporo, Japan) were analyzed in this study. Significant differences were found between the hERO1-α-positive group ($n = 33$) and hERO1-α-negative group ($n = 38$) in nuclear grade ($P < 0.001$) and intrinsic subtype ($P = 0.021$) in univariate analysis. More importantly, in multivariate analysis of disease-free survival by Cox regression, expression of hERO1-α was the only independent prognosis factor ($P = 0.035$). Finally, in univariate survival analysis, patients positive for hERO1-α had significantly shorter disease-free survival and overall survival than those patients negative for hERO1-α. These findings indicate that the expression of hERO1-α in cancer cells is associated with poorer prognosis and thus can be a prognostic factor for patients with breast cancer. (*Cancer Sci* 2013; 104: 1091–1096)

Hypoxia is a physiologically important characteristic that is present in all tumors. Importantly, tumor hypoxia exerts a pronounced effect on malignant progression and metastatic spread of human cancers.[1] Numerous clinical studies have shown that tumor hypoxia predicts decreased local control, increased distant metastases, and decreased overall survival in a variety of human tumors. Hypoxia has been shown to select tumors with an augmented malignant phenotype and increase the metastatic potential of tumor cells.[1,2] It is known that hypoxic cells are relatively resistant to killing by radiation.[3] In addition, because hypoxic cells are non-proliferating and relatively isolated from the blood supply, chemotherapies that target rapidly dividing cells may be less effective for this population of cells and the delivery of chemotherapy to these areas may be compromised. Thus, tumor hypoxia correlates with a more aggressive disease course and limits the effectiveness of anticancer therapy.[4] Human endoplasmic reticulum oxidoreductin 1-α (hERO1-α) is a hypoxia-inducible endoplasmic reticulum-resident oxidase that regulates the post-translational branch of oxidized protein folding.[5,6] It has been identified as a reoxidizer of protein disulfide isomerase (PDI),[7,8] which functions as a disulfide-introducing enzyme for secretory and cell-surface molecules in the cell.[9] Therefore, PDI exists mainly as an oxidized form within a cell. Human ERO1-α is expressed in normoxic cells at very low level. However, it has been shown that hERO1-α is induced in hypoxic cells in response to low oxygen availability.[6] Importantly, the expression level of PDI was also shown to be augmented under hypoxic conditions,[10] suggesting that hERO1-α plays a key role in hypoxic cancer cells in concert with PDI. Although it has been shown that hERO1-α plays a pivotal role in hypoxia-inducible factor 1-mediated vascular endothelial growth factor (VEGF) production,[11] the role of hERO1-α in *in vivo* tumor growth has yet to be elucidated. Many genes associated with breast cancer metastasis have been reported to be upregulated under hypoxic conditions, and hypoxic gene signatures are associated with poor outcome in breast cancer.[12,13]

In this study, we investigated the effect of hERO1-α on tumor progression using the murine ERO1-α-positive metastatic murine breast cancer cell line 4T1. We also investigated the effects of knocking down murine ERO1-α with siRNA. We further investigated the expression of hERO1-α and its clinical relevance in 71 breast cancer patients with long-term follow-up by immunohistochemistry. The expression of estrogen receptor (ER) and nuclear grade were also investigated. We found that a high expression level of hERO1-α in breast cancer tissues was associated with nuclear grade status and was an independent prognostic factor for breast cancer patients after surgery. Thus, understanding the relationships between hypoxia, hERO1-α expression, and tumor growth is crucial for improving current breast cancer therapies.

Materials and Methods

Cell and cell culture under hypoxic conditions. Murine breast cancer cell line 4T1 was purchased from ATCC (Manassas, VA, USA). Cells were cultured in RPMI-1640 (Nissui, Tokyo, Japan) supplemented with 10% FCS. Short hairpin RNA for murine ERO1-α (TR502816) was purchased from OriGene (Rockville, MD, USA) and transfected to 4T1 cells using Lipofectamine RNAiMAX (Life Technologies, Carlsbad, CA, USA). The 4T1 cells were cultured under hypoxic conditions (1% O_2, 5% CO_2, and 94% N_2) for at least 3 days. Cells maintained under normoxic conditions (20% O_2, 5% CO_2, and 75% N_2) were used as controls.

Reverse transcription–PCR analysis. Total RNA was isolated from cultured cells, breast cancer tissues, and normal breast tissues using Isogen reagent (Nippon Gene, Tokyo, Japan). The cDNA mixture was synthesized from 1 μg total RNA by reverse transcription using Superscript III and oligo (dT) primer (Life Technologies) according to the manufacturer's protocol. The PCR amplification was carried out in 50 μL PCR mixture containing 1 μL cDNA mixture, KOD Plus DNA polymerase

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(Toyobo, Osaka, Japan), and 50 pmol primers. The PCR mixture was initially incubated at 92°C for 2 min, followed by 30 cycles of denaturation at 92°C for 1 min, annealing at 62°C for 1 min, and extension at 72°C for 1 min. Primer pairs used for RT-PCR analysis were 5′-GCCCGTTTTATGCTTGATGT-3′ and 5′-AACTGGGTATGGTGGCAGAC-3′ for human *ERO1-α*. As an internal control *G3PDH* was detected by using the forward primer 5′-ACCACAGTCCATGCCATCAC-3′ and the reverse primer 5′-TCCACCACCCTGTTGCTGTA-3′.

Western blot analysis. Cultured cells were washed in ice-cold PBS, lysed by incubation on ice in a lysis buffer (50 mmol/L Tris-HCl [pH 8.0], 150 mmol/L NaCl, 1% NP40, protease inhibitor cocktail; Complete [Roche Diagnostics, Basel, Switzerland]), and cleared by centrifugation at 21880*g* for 20 min at 4°C. For blockade of free thiols, cells were pretreated for 5 min with 10 mM methyl methanethiosulfonate (Pierce, Rockford, IL, USA) in PBS. Cells were lysed in 1% NP40 in TBS buffer containing 25 mM HEPES, 100 mM NaCl, 10 mM $CaCl_2$, and 5 mM $MgCl_2$ (pH 7.6) supplemented with a protease inhibitor and 5 mM methyl methanethiosulfonate. Post-nuclear supernatants were divided and heated for 5 min at 95°C in non-reducing or reducing SDS sample buffer, resolved by 10% SDS-PAGE, and electrophoretically transferred to PVDF membranes (Immobilon-P; Millipore, Billerica, MA, USA). The membranes were incubated with blocking buffer (5% non-fat dried milk in PBS) for 1 h at room temperature then incubated for 60 min with anti-ERO1-α mAb (Abnova, Taipei, Taiwan), anti-PDI polyclonal antibody (Enzo Life Sciences, Farmingdale, NY, USA) or mouse anti-β-actin mAb AC-15 (Sigma-Aldrich, St. Louis, MO, USA). After washing three times with wash buffer (0.1% Tween-20 in PBS), the membranes were reacted with peroxidase-labeled goat anti-rabbit IgG antibody (KPL, Gaithersburg, MD, USA) for 2 h. Finally, the signal was visualized using an ECL detection system (Amersham Life Science, Arlington Heights, IL, USA) according to the manufacturer's protocol.

Proliferation assay. A cell proliferation assay based on cleavage of the tetrazolium salt WST-1 (DOJINDO, Kumamoto, Japan) by mitochondrial dehydrogenases in viable cells was carried out according to the manufacturer's instructions. The cells were seeded on 96-well microtiter tissue culture plates in 10% serum at a density of 1×10^3 cells/well. After incubation at 37°C for 24–72 h, WST-1 reagent (10 μL) was added to the cells and the cells were incubated at 37°C in 96-well microtiter tissue culture plates for 2 h at 37°C. The amount of formazan dye produced, which directly correlates to the number of metabolically active cells in culture, was quantified by measuring the absorbance at a wavelength of 450 nm using a microtiter plate (ELISA) reader.

***In vivo* studies.** Female BALB/c mice, 5–6 weeks old, were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and used at 6 weeks of age. Mice were maintained in a specific pathogen-free mouse facility at Sapporo Medical University (Sapporo, Japan) according to institutional guidelines for animal use and care. For tumor formation studies, mice were injected with 1×10^5 4T1 or ERO1-α knockdown cells into right mammary glands. Tumor length and width were measured with a caliper. All of the experiments were carried out with five mice /group. Average tumor diameters on day 42 were statistically analyzed using the Mann–Whitney *U*-test. At day 42, numbers of lung metastases were counted and compared.

Enzyme-linked immunosorbent assay. The 4T1 cells were plated at 1×10^4 cells/well in flat-bottomed, 96-well plates for 24 h. Supernatants were diluted and tested for mouse VEGF-A (R&D Systems, Minneapolis, MN, USA) using a sandwich ELISA kit. Absorbance was determined at 450 nm.

Patients and immunohistochemical variables for specimens. Tissue samples were obtained from 71 patients diagnosed with breast cancer in 2005 at Sapporo Medical University Hospital. A total of 71 specimens of primary invasive carcinoma were obtained from resected tumors. All of the specimens used in this study were fixed in neutral 10% buffered formaldehyde, embedded in paraffin, and cut into 5-μm slices. Other background data for the patients are shown in Table 1. The expression of estrogen receptor (ER), progesterone receptor (PgR), human epidermal growth factor receptor type 2 (HER2), and hERO1-α was determined immunohistochemically in paraffin-embedded tissue specimens. Monoclonal Abs against ER, PgR, and HER2 were purchased from DAKO Japan (Tokyo, Japan). The expression of ER or PgR was designated as positive when at least 10% of the tumor nuclei showed positive staining. The expression of HER2 was classified according to the Hercep Test assay's scoring system, which includes four categories, 0, 1+, 2+ and 3+, based on the intensity and proportion of membrane staining in tumor cells. Positivity was defined as a HER2 score of 3+ for immunostaining or >2-fold increase in HER2 gene amplification, as determined by FISH. The expression of hERO1-α was designated as positive when at least 30% of perinuclear staining within tumor cells was observed. Histological examinations were carried out on slides with paraffin-embedded samples stained by H&E according to the criteria of the Japanese Breast Cancer Society, which are based on the International Union against Cancer TMN classification criteria.

Statistical analysis. The χ^2-test and unpaired *t*-test were used for analysis of two unpaired samples. Disease-free survival and overall survival rates after surgical resection were calculated by the Kaplan–Meier method, and differences in survival curves were assessed by the log–rank test. The Cox proportional hazards model was used for multivariate analysis. All analyses were carried out with SPSS version 18.0 (SPSS Inc.,

Table 1. Background data for patients with breast cancer who participated in this study (*n* = 71)

	n	%
Mean age, years (range)	56	(25–82)
Histological type		
Papillotubular	28	39.4
Solid-tubular	7	9.9
Scirrhous	25	35.2
Others	11	15.5
pT		
pT1 (≤ 2.0 cm)	22	31.0
pT2 (2.0 ≤ 5.0 cm)	43	60.6
pT3 (>5.0 cm)	6	8.4
pN		
pN (–)	47	66.2
pN (+)	24	33.8
ER, PgR, HER2 status		
ER (+) or PgR (+) and HER2 (–)	42	59.1
ER (+) or PgR (+) and HER2 (+)	10	14.1
ER (–) and PgR (–) and HER2 (+)	11	15.5
ER (–) and PgR (–) and HER2 (–)	8	11.3
Nuclear grade (NG)		
NG1	22	31.0
NG2	27	38.0
NG3	22	31.0
hERO1-α		
(+)	33	46.5
(–)	38	53.5

ER, estrogen receptor; HER2, human epidermal growth factor receptor type 2; hERO1-α, human endoplasmic reticulum oxidoreductin 1-α; PgR, progesterone receptor.

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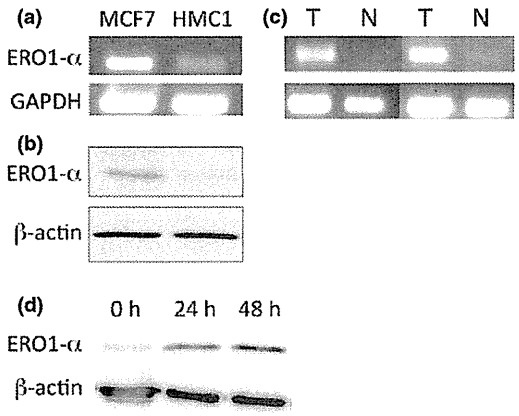

Fig. 1. Expression of human endoplasmic reticulum oxidoreductin 1-α (hERO1-α) in human breast cancer cell lines and tissue samples. (a) Human ERO1-α mRNA levels in MCF7 and HMC1 cells determined by RT-PCR analysis. (b) Western blot analysis of MCF7 and HMC1 cells. (c) mRNA expression of hERO1-α in breast cancer tissues (T) and normal breast tissues (N). (d) Induction of ERO1-α under hypoxic conditions. Western blot analysis of murine breast cancer cell line 4T1 cultured under hypoxic conditions for indicated periods (0, 24 and 48 h).

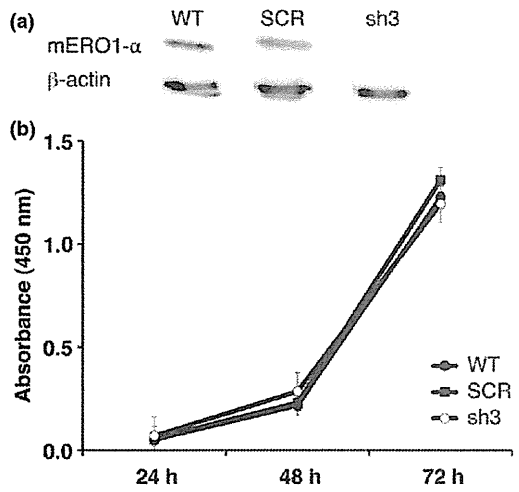

Fig. 2. Functional analysis after endoplasmic reticulum oxidoreductin 1-α (ERO1-α) knockdown in murine breast cancer 4T1 cells. (a) Establishment of a murine ERO1-α (mERO1-α)-depleted 4T1 cell clone by shRNA transfection. ERO1-α was decreased significantly according to Western blot analysis. (b) Cell proliferation was compared by WST-1 assay. Mean ± SD from individual experiments with three replicate assays. Three independent clones were tested and representative data are shown. SCR, 4T1 cell transfected with short hairpin RNA for scramble control; sh3, 4T1 cell clone transfected with shRNA for mERO1-α.

Chicago IL, USA). A P-value of less than 0.05 was regarded as statistically significant. All statistical tests were two-sided.

Results

Human ERO1-α expression in breast cancer cell lines and breast cancer tissues. Expression of hERO1-α was detected in the human breast cancer cell line MCF-7 and to a lesser extent in HMC1 cells by RT-PCR (Fig. 1a). Although the expression level of hERO1-α protein was high in MCF7 cells, the protein level of hERO1-α was low in HMC1 cells (Fig. 1b). Interestingly, mRNA of ERO1-α was observed in breast cancer tissues

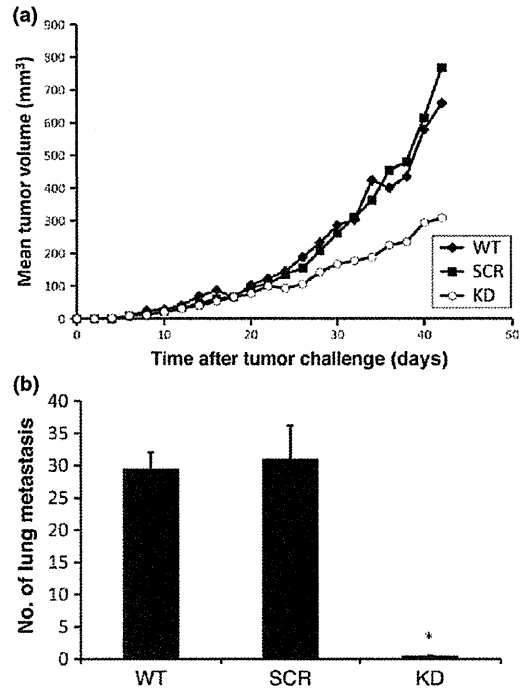

Fig. 3. Expression of endoplasmic reticulum oxidoreductin 1-α (ERO1-α) significantly affects tumor growth and lung metastasis. Female BALB/c mice (five animals/group) were injected with 1×10^5 murine breast cancer 4T1 cells (WT), short hairpin RNA for scramble control (SCR), or ERO1-α knockdown cell (KD) into right mammary glands. At day 42, numbers of lung metastases were counted. Tumor growth (a) and lung metastasis (b) of 4T1 (WT), SCR, and KD were compared. Representative data are shown of four independent experiments. *$P < 0.005$, paired Student's t-test.

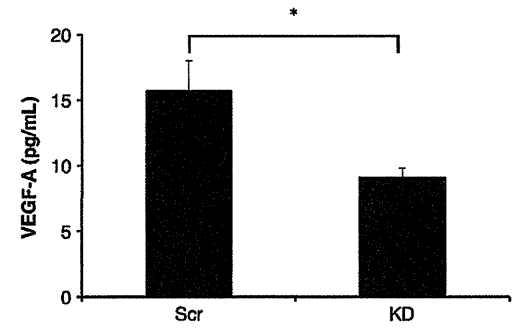

Fig. 4. Endoplasmic reticulum oxidoreductin 1-α (ERO1-α) regulates vascular endothelial growth factor-A (VEGF-A) production from murine breast cancer 4T1 cells. Concentration of VEGF-A in the 24-h culture supernatant from 4T1 cells transfected with short hairpin RNA for scramble control (Scr) or 4T1 cells transfected with short hairpin RNA for mERO1-α (KD) was measured using ELISA. Representative data are shown of three independent experiments. *$P < 0.01$, paired Student's t-test.

but not in normal mammary gland tissues (Fig. 1c). Notably, the expression of ERO1-α was augmented in 4T1 cells in response to hypoxic conditions, suggesting that tumor hypoxia induces the expression of ERO1-α (Fig. 1d).

Knockdown of ERO1-α by shRNA reduced tumor growth and metastasis. To further examine the functional role of ERO1-α in breast cancer cells, murine breast cancer cell line 4T1 cells were transfected with an shRNA vector targeting murine

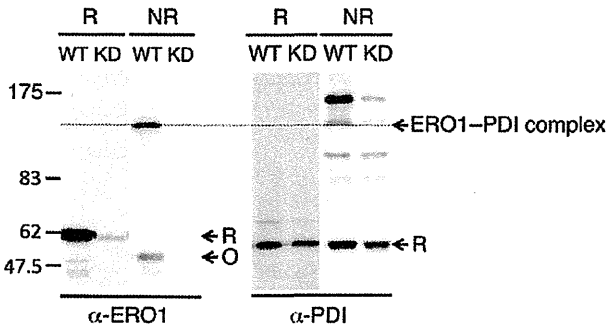

Fig. 5. Endoplasmic reticulum oxidoreductin 1-α (ERO1-α) forms a mixed disulfide linkage with protein disulfide isomerase (PDI) and effects of ERO1-α depletion on the redox status of PDI. Redox status of ERO1-α and PDI in murine breast cancer 4T1 cells (WT) or ERO1-α-depleted cells (KD) was examined by Western blotting under reducing (Red) or non-reducing (NR) conditions. Reduced form (R) or oxidized form (O) of ERO1-α or PDI are indicated. Note that depletion of ERO1-α decreased the ERO1-α–PDI complex.

ERO1-α. In 4T1 cells, successful knockdown of murine ERO1-α expression was confirmed by Western blot analysis (Fig. 2a). The 4T1 cells with downregulation of ERO1-α by shRNA did not show the differences in proliferation assay compared with WT and control shRNA-transfected cells (Fig. 2b). In contrast, knockdown of ERO1-α caused retardation of *in vivo* tumor growth compared with WT 4T1 (Fig. 3a). The number of lung metastases of ERO1-α knockdown 4T1 cells was also significantly decreased compared with that of WT cells (Fig. 3b). These results suggested that the expression of ERO1-α accelerated tumor growth and lung metastasis. To explore the role of ERO1-α in tumor progression and augmented metastasis, we compared VEGF-A production from 4T1 cells transfected with short hairpin RNA for scramble control cells and that from ERO1-α knockdown cells, because VEGF-A is a homodimer whose proper folding through formation of three intramolecular disulfide bonds and two intersubunit disulfide bonds is a prerequisite for its function.[14–16] We found that knockdown of ERO1-α decreased the production of VEGF-A (Fig. 4). These results suggested that tumor hypoxia drove VEGF-A production from tumor through the function of ERO1-α. Thus, ERO1-α plays an important role in tumor growth and metastasis via its upregulation under the condition of tumor hypoxia.

Endoplasmic reticulum oxidoreductin 1-α forms a mixed disulfide linkage with PDI. The formation of native protein disulfide bonds is a critical step in the folding of many secretory and cell-surface proteins. Protein disulfide isomerase serves as a principal catalyst of thiol-disulfide exchange in the lumen of the endoplasmic reticulum. Disulfide transfer to the substrate protein by PDI will result in reduction of the active site of PDI, which must be reoxidized to carry out further oxidation.[7] Mixed disulfide complexes between ERO1-α and PDI have been demonstrated, and these complexes are likely to represent intermediates in the direct oxidation of PDI by ERO1-α *in vivo*.[17] Therefore, we examined whether knockdown of ERO1-α affect the redox status of PDI. Western blot analysis revealed that the ERO1-α–PDI mixed disulfides migrated with an apparent molecular mass of approximately 140 kDa under non-reducing conditions (Fig. 5). Knockdown of ERO1-α in 4T1 cells decreased the ERO1-α–PDI complex, indicating that depletion of ERO1-α decreased the oxidized form of PDI. These data suggested that depletion of ERO1-α decreased disulfide bond formation in VEGF-A, resulting in decreased VEGF-A secretion. Thus, ERO1-α clearly affects the redox status of PDI and production of secreted protein including VEGF-A.

Prognostic factors. Next, we examined whether expression of hERO1-α had an impact on the clinical course of human breast cancer. The expression of hERO1-α was investigated by immunohistochemistry using anti-ERO1-α mAb. Normal breast tissues including mammary ducts revealed negative staining for ERO1-α (Fig. 6a). These results are in good agreement with RT-PCR analysis (Fig. 1c). Thirty-eight cases (53.5%) of the 71 patients with breast cancer showed negative for hERO1-α (Fig. 6b). In contrast, perinuclear staining of

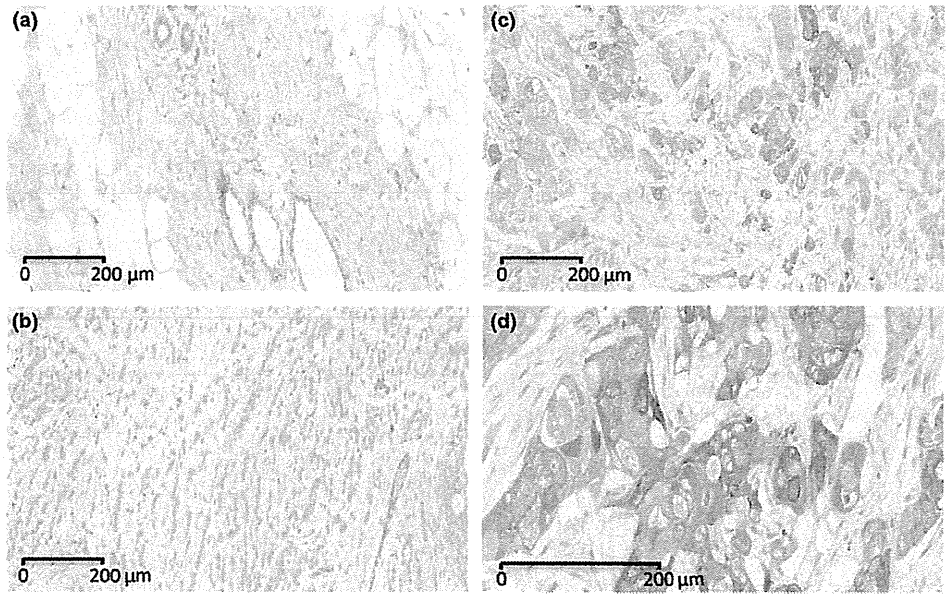

Fig. 6. Expression of human endoplasmic reticulum oxidoreductin 1-α (hERO1-α) in normal breast tissue and breast cancer tissue by immunohistochemical analysis. (a) Normal breast tissue (×200). (b) Breast cancer tissue negative for hERO1-α (×200). (c, d) Perinuclear staining for hERO1-α, indicating endoplasmic reticulum localization in breast cancer cells (c, ×40; d, ×200).

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