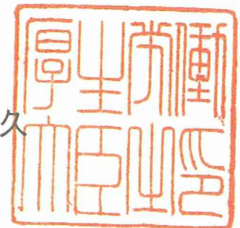
〔資料３〕

厚生労働省発科０３０４第２号
平成２６年３月４日

岡山大学病院
病院長　槇野博史　殿

厚生労働大臣　田村憲久



遺伝子治療臨床研究実施計画について

平成25年8月8日付けで申請のあった下記の臨床研究については、実施して差し支えない。

なお、臨床研究の中止、終了等に伴う厚生労働大臣への報告については、遺伝子治療臨床研究に関する指針及び関係通知の定めるところによるほか、定期的に中間報告書を提出するようお願いする。

記

課題名：悪性胸膜中皮腫に対する Reduced Expression in Immortalized Cells/Dickkopf-3 遺伝子発現アデノウイルスベクターを用いた遺伝子治療臨床研究

総括責任者：豊岡　伸一（岡山大学大学院医歯薬学総合研究科臨床遺伝子医療学教授）

科発０３０４第２号
平成２６年３月４日

岡山大学病院
病院長　槇　野　博　史　　殿

厚生労働省大臣官房厚生科学課長

遺伝子治療臨床研究実施計画について

平成25年8月8日付けで申請のあった遺伝子臨床研究実施計画については、平成26年3月4日厚生労働省発科0304第2号により厚生労働大臣から実施して差し支えない旨通知されたところであるが、当該臨床研究の実施に当たっては、下記に留意して適切に実施するようお願いする。

記

１　「遺伝子治療臨床研究に関する指針」
（平成14年文部科学省・厚生労働省告示第1号、全部改正平成16年文部科学省・厚生労働省告示第2号、一部改正平成21年文部科学省・厚生労働省告示第2号）

２　「遺伝子治療臨床研究に関する指針について」
（平成14年3月27日付け13文科振第1144号・科発第0327001号文部科学省研究振興局長及び厚生労働省大臣官房厚生科学課長連名通知）

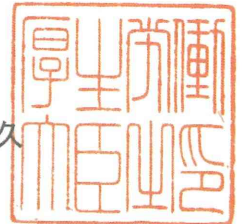
３　「遺伝子治療臨床研究に関する指針の改訂等について」
（平成16年12月28日付け16文科振第931号・科発第1228003号文部科学省研究振興局長及び厚生労働省大臣官房厚生科学課長連名通知）

（参考）
http://www.mhlw.go.jp/general/seido/kousei/i-kenkyu/index.html#3

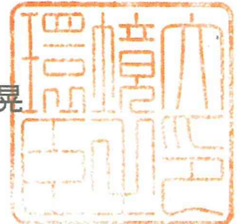
厚生労働省発科０３０４第５号
環自野発第 1403041 号
平成２６年３月４日

岡山大学病院
病院長　槇野　博史　殿

厚生労働大臣　田　村　憲　久



環境大臣　石　原　伸　晃



遺伝子組換え生物等の使用等の規制による生物の多様性の確保に関する法律に基づく第一種使用規程の承認について（通知）

平成 25 年 8 月 8 日付けで承認申請のあった第一種使用規程について、遺伝子組換え生物等の使用等の規制による生物の多様性の確保に関する法律（平成 15 年法律第 97 号）第 4 条第 1 項の規定に基づき、別紙のとおり承認したので通知する。

（別紙）

承認番号　14－36Ｖ－0002

遺伝子組換え生物等の種類の名称	Ｒｅｄｕｃｅｄ　Ｅｘｐｒｅｓｓｉｏｎ　ｉｎ　Ｉｍｍｏｒｔａｌｉｚｅｄ　Ｃｅｌｌｓ／Ｄｉｃｋｋｏｐｆ－３（ＲＥＩＣ／Ｄｋｋ－３）遺伝子を発現する非増殖性の遺伝子組換えヒトアデノウイルス５型（Ａｄｖ／ｈＲＥＩＣ）
遺伝子組換え生物等の第一種使用等の内容	治療施設におけるヒトの治療を目的とした使用、保管、運搬及び廃棄並びにこれらに付随する行為
遺伝子組換え生物等の第一種使用等の方法	治療施設の所在地　岡山県岡山市北区鹿田町二丁目５番１号 治療施設の名称　岡山大学病院 (1)　Ａｄｖ／ｈＲＥＩＣ溶液は、容器に密封後、凍結状態で治療施設に輸送し、施設内のＰ２レベルの実験室（以下「Ｐ２実験室」という。）内の冷凍庫に保管する。 (2)　凍結状態のＡｄｖ／ｈＲＥＩＣ溶液の融解、希釈及び分注操作は、Ｐ２実験室内の安全キャビネット内で行う。Ａｄｖ／ｈＲＥＩＣ希釈溶液の保管は、Ｐ２実験室内の冷凍庫において行う。なお、Ａｄｖ／ｈＲＥＩＣ希釈溶液又はその凍結品を開放系区域を通って他のＰ２レベル区域に運搬する必要がある場合には、二重に密閉した容器に入れて運搬する。 (3)　Ａｄｖ／ｈＲＥＩＣ溶液（希釈溶液を含む。）を廃棄する際には、ウイルスの不活化(0.18%若しくは0.24%次亜塩素酸ナトリウム溶液による消毒薬処理又は高圧蒸気滅菌処理による。以下同じ。）を行った後、岡山大学病院で定められた医療廃棄物管理規程（以下単に「医療廃棄物管理規程」という。）に従い廃棄する。 (4)　Ｐ２実験室内の安全キャビネット内でＡｄｖ／ｈＲＥＩＣ溶液を緩衝液で希釈し、所定の投与量に調整したもの（以下「Ａｄｖ／ｈＲＥＩＣ液」という。）を、二重に密閉し、岡山大学病院総合診療棟ＩＶＲ－ＣＴ室（以下「ＣＴ室」という。）に直ちに運搬し、専用の注入用穿刺針、注射器及びチューブから成るデバイス（以下「注入セット」という。）に充填する。 (5)　悪性胸膜中皮腫に罹患した被験者に対するＡｄｖ／ｈＲＥＩＣ液の投与は、ＣＴ室において、局所麻酔下で、Ａｄｖ／ｈＲＥＩＣ液をあらかじめ留置している胸腔内チューブを用いて、又はＣＴガイド下に注入用穿刺針を用いて、胸水貯留を認める胸腔内又は評価可能な１病変部に注入することにより行う。注入針の抜去は慎重に行い、Ａｄｖ

	／ｈＲＥＩＣ液の漏出及びエアロゾル化を防止する。注入部位の周辺には、滅菌された不織布を二重に敷き詰める。 (6) 被験者へのＡｄｖ／ｈＲＥＩＣ液の投与終了後、被験者の創部を消毒する。ウイルス漏出予防のためにマスク及びガウンを着用した被験者を、ＣＴ室から、環境中への拡散防止措置を適切に執った陽圧でない個室（以下単に「個室」という。）に移送する。 (7) (5)及び(6)で用いた注入セット等の器具、布及びガーゼ類は、ウイルスの不活化を行い、医療廃棄物管理規程に従い廃棄する。また、穿刺用ガイド装置等は、ウイルスの不活化を行い、再利用する。これらのウイルスの不活化をＣＴ室以外の区域で行う場合には、二重に密閉した容器に入れて運搬する。治療後の治療室は床を消毒液で掃き清掃する。なお、治療室内の空気はＨＥＰＡフィルターを用いた換気により約５分に１回（１時間に 12 回）入れ替える。 (8) Ａｄｖ／ｈＲＥＩＣ液の投与後24時間､被験者を個室内で管理する。また、Ａｄｖ／ｈＲＥＩＣ液を胸腔内に注入する際に胸腔内カテーテルチューブを挿入した場合は、その抜去後 24 時間、又は被験者より排出された胸水中のＡｄｖ／ｈＲＥＩＣが陰性であることが確認できるまで、被験者を個室内で管理する。検査等の理由で被験者が一時的に個室から外の開放系区域に出る場合には、採血や排泄等を最小限に留め、マスク及びガウン着用等のウイルス漏出予防措置を義務付ける。 (9) 個室における管理期間中の被験者の排泄物等（血液、体液、尿及び糞便等をいう。以下同じ。）は、ウイルスの不活化を行った後、医療廃棄物管理規程に従い廃棄する。ウイルスの不活化を個室以外の区域で行う場合には、二重に密閉した容器に入れて運搬する。なお、研究用検体として使用する被験者の排泄物等の取扱いは、Ａｄｖ／ｈＲＥＩＣ溶液の取扱いに準ずる。排泄物等が床等に落下した場合は床等を消毒液で掃き清掃する。 (10) 個室における管理期間中、被験者に対して侵襲的に使用した器具等及び被験者の排泄物等に接触した器具等は、ウイルスの不活化を行った後、医療廃棄物管理規程に従い廃棄するか、又は十分洗浄する。ウイルスの不活化を個室以外の区域で行う場合には、二重に密閉した容器に入れて運搬する。 (11) 個室における被験者の管理を終了する前に、被験者の血液及び尿中のＡｄｖ／ｈＲＥＩＣが陰性であることを確認する。Ａｄｖ／ｈＲＥＩＣが確認されたときは、個室における被験者の管理を継続する。ま

	た排泄物等の床等への落下の有無にかかわらず、個室における管理終了後は、床等を消毒液で掃き清掃する。 ⑿　個室における被験者の管理の終了後に、遺伝子治療臨床研究実施計画書（悪性胸膜中皮腫に対するＲｅｄｕｃｅｄ　Ｅｘｐｒｅｓｓｉｏｎ　ｉｎ　Ｉｍｍｏｒｔａｌｉｚｅｄ　Ｃｅｌｌｓ／Ｄｉｃｋｋｏｐｆ－３遺伝子発現アデノウイルスベクターを用いた遺伝子治療臨床研究に関する実施計画書）に示す観察期間内に被験者の血液又は尿中からＡｄｖ／ｈＲＥＩＣが検出された場合には、直ちに被験者を個室における管理下に移し、⑻から⑾までと同様の措置を執る。

Ⅱ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

書籍

該当なし

著者氏名	論文タイトル名	書籍全体の編集者名	書 籍 名	出版社名	出版地	出版年	ページ

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Jin Y., Murata H., Sakaguchi M., Kataoka K., Watanabe M., Nasu Y., Kumon H., Huh NH.	Partial sensitization of human bladder cancer cells to a gene-therapeutic adenovirus carrying REIC/Dkk-3 by downregulation of BRPK/PINK1.	Oncol Rep	27（3）	695-699	2012
Ochiai K, Watanabe M, Azakami D, Michishita M, Yoshikawa Y, Udagawa C, Metheenukul P, Chahomchuen T, Aoki H, Kumon H, Morimatsu M, Omi T.	Molecular cloning and tumour suppressor function analysis of canine REIC/Dkk-3 in mammary gland tumours.	Vet J.	197（3）	769-75	2013
Shien K, Tanaka N, Watanabe M, Soh J, Vet J. Sakaguchi M, Matsuo K, Yamamoto H, Furukawa M, Asano H, Tsukuda K, Nasu Y, Huh NH, Miyoshi S, Kumon H, Toyooka S.	Anti-cancer effects of REIC/Dkk-3-encoding adenoviral vector for the treatment of non-small cell lung cancer.	PLoS One.	9（2）	e87900	2014

Ⅲ. 研究成果の刊行物・別冊

ONCOLOGY REPORTS 27: 695-699, 2012

Partial sensitization of human bladder cancer cells to a gene-therapeutic adenovirus carrying REIC/Dkk-3 by downregulation of BRPK/PINK1

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Abstract. REIC/Dkk-3 is a tumor suppressor gene that was first identified as a gene downregulated in association with immortalization of normal human fibroblasts. We have demonstrated that an adenovirus carrying REIC/Dkk-3 (Ad-REIC) showed a tumor-specific killing effect on a wide range of cancers. However, some human cancers, bladder cancers in particular, are resistant to Ad-REIC. In this study, we investigated the combination effect of downregulation of BRPK/PINK1 (PINK1) and Ad-REIC on bladder cancer cells. Five bladder cancer cell lines among six cell lines examined were resistant to Ad-REIC. Among the cell lines, the resistance of two cell lines was probably due to low infection efficiency of the adenovirus. PINK1-specific siRNA remarkably downregulated Bcl-x_L and TRAP1 proteins and upregulated BAX protein expression. Finally, downregulation of PINK1 partially sensitized the other three cell lines that were resistant to Ad-REIC. This sensitization was associated with increasing production of reactive oxygen species (ROS). These results indicate that PINK1 is one of the key molecules for the mitochondrial protection system and that PINK1 can be a new target molecule to sensitize bladder cancer cells that are resistant to Ad-REIC.

Introduction

REIC/Dkk-3 is a tumor suppressor gene that was first identified as a gene downregulated in association with immortalization of normal human fibroblasts (1). Subsequently, we found that overexpression of REIC/Dkk-3 using an adenovirus vector (Ad-REIC) showed a tumor-specific killing effect on a wide range of cancers, including those derived from the prostate, testis, pleura, breast and stomach (2-6). However, some human cancers, bladder cancers in particular, are resistant to Ad-REIC partly because of high expression levels of mitochondrial anti-apoptotic proteins such as Bcl-2 and Bcl-x_L (7). Upon initiation of apoptotic signaling, the pro-apoptotic Bcl-2 protein BAX undergoes a conformation shift and is inserted into the outer mitochondrial membrane, which increases membrane permeability (8,9). This results in the release of cytochrome c and other pro-apoptotic factors from the mitochondria, eventually leading to apoptosis. In contrast, Bcl-2 and Bcl-x_L are anti-apoptotic proteins and inhibit the release of pro-apoptotic factors from the mitochondria (10). Thus, the ratio between pro- and anti-apoptotic proteins of the Bcl-2 family is one of the critical determining factors for apoptosis, and such proteins function on the mitochondria.

The BRPK/PINK1 (PINK1) gene encodes a serine/threonine kinase with a mitochondrial localization signal, and mutations of the gene are associated with autosomal recessive inheritance of Parkinson's disease (11). Overexpression of wild-type PINK1 protected neuronal cells against various stresses (12), whereas downregulation of PINK1 sensitized neuroblastoma cells to various stresses (13). On the other hand, we showed that PINK1 was expressed at high levels in malignant cancer cells exhibiting an increased metastatic activity (14) and that PINK1 protected cancer cells against various cytotoxic agents through Akt activation (15,16). Martin *et al* (17) reported that PINK1 is a potential therapeutic target for the treatment of DNA mismatch repair-deficient cancers. Thus, accumulating lines of evidence indicate that PINK1 protects cancer cells from stress-mediated mitochondrial dysfunction, various stresses and apoptosis. In the present study, we examined the possibility that downregulation of PINK1 may sensitize bladder cancer cells that are resistant to Ad-REIC through inducing mitochondrial dysfunction.

Materials and methods

Cell culture. Human bladder cancer cell lines T24, J82, 5637, UM-UC-3 and TCCSUP were purchased from ATCC

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Key words: REIC/Dkk-3, gene therapy, bladder cancer, BRPK/PINK1, sensitization

(Rockville, MD). The human bladder cancer cell line KK47 (18) was a gift from Dr Kumazawa (Department of Urology, Faculty of Medicine, Kyushu University). These cells were cultured in DMEM/F12 (Ham) (1:1) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum.

Adenovirus vectors and infection. Ad-REIC was produced and propagated as previously described (2). An adenovirus vector carrying the LacZ gene (Ad-LacZ) was used for monitoring infection efficiency. Twenty-four hours after subculture, cells were treated with Ad-LacZ or Ad-REIC at the indicated multiplicity of infection (MOI).

Apoptosis assay. Apoptotic cells were identified after staining with 0.2 μM Hoechst 33342 (Invitrogen) for 30 min. Under a fluorescence microscope, cells with fragmented or shrunk nuclei were counted as apoptotic cells.

Assay for adenovirus infection efficiency. To determine adenovirus infection efficiency, cells were infected with Ad-LacZ. Forty-eight hours after infection, the cells were washed with PBS, fixed at 4°C for 10 min with 0.25% glutar aldehyde, rinsed 4 times with PBS, and then stained with 2.5 mM 5-bromo-4-chloro-3-indolyl-D-galactoside (X-Gal) at 37°C for 18 h.

Western blot analysis. Western blot analysis was performed under conventional conditions after lysing cells with M-PER mammalian protein extraction reagent (Thermo Scientific) with PhosSTOP Phosphatase Inhibitor (Roche Applied Science). The antibodies used were as follows: antibody against PINK1 (Novus); antibodies against BiP, TRAP1, Bcl-x_L, and BAX (BD Pharmingen, San Jose, CA); antibodies against Grp75 and Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA); antibody against β-actin (Sigma); and HRP-labeled anti-mouse and anti-rabbit secondary antibodies (Cell Signaling Technology, Danvers, MA).

RT-PCR. Total RNA was extracted with the SV Total RNA Isolation system (Promega, Madison, WI). The Total RNA was used to synthesize cDNA with the Superscript III First-Strand Synthesis system (Invitrogen). RT-PCR was performed under conventional conditions. The primers used were as follows: human PINK1 (forward) 5'-CACCTTGAAAGCC GCAGCTACCAAGA-3', human PINK1 (reverse) 5'-AGC AGAGGAGGGCTGCCT-3', human Bcl-x_L (forward) 5'-CCA CCTAGAGCCTTGGATCCA-3', human Bcl-x_L (reverse) 5'-ACGCCGGCCACAGTCATG-3', human Bcl-2 (forward) 5'-CCCTGGTGGACAACATCGC-3', human Bcl-2 (reverse) 5'-CCAGGAGAAATCAAACAGAGGC-3', human glyceraldehyde-3-phosphate dehydrogenase (GAPDH: forward) 5'-ATTCCATGGCACCGTCAAGGCT-3', human GAPDH (reverse) 5'-TCAGGTCCACCACTGACACGTT-3'.

RNA interference. siGENOME SMARTpool siRNA targeting PINK1 (NM_032409) (Thermo Scientific Dharmacon, Lafayette, CO) was transfected into cells using Lipofectamine RNAi MAX (Invitrogen). A control siRNA with no known mammalian homology (siGENOME non-targeting siRNA pool 1, Thermo Scientific Dharmacon) was used as a negative control.

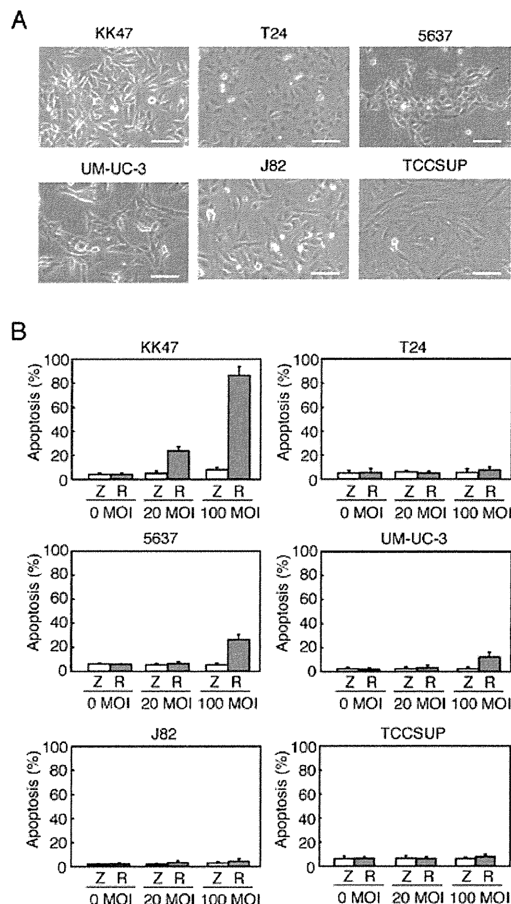

Figure 1. Resistance of bladder cancer cell lines to Ad-REIC-induced apoptosis. (A) Morphology of human bladder cancer cell lines. Bars, 100 μm. (B) Rates of apoptosis induction by Ad-REIC in bladder cancer cell lines. Cells were infected with an adenovirus vector carrying REIC (R) or LacZ (Z). Fourty-eight hours after the infection, apoptotic cells were determined by staining with Hoechst 33342.

Assay for reactive oxygen species (ROS) formation. To visualize intracellular ROS, BES-H_2O_2 staining was performed. Cells were incubated with 1 μM BES-H_2O_2 (Wako Chemicals, Osaka, Japan) at 37°C for 1 h. After washout by rinsing thoroughly, BES-H_2O_2 signal was detected by 488 nm excitation.

Results

Resistance of human bladder cancer cell lines to Ad-REIC-induced apoptosis. At first, we examined the sensitivity to Ad-REIC using 6 bladder cancer cell lines, KK47, T24, 5637, UM-UC-3, J82 and TCCSUP (Fig. 1A). Among those cell lines, only KK47 was sensitive to the induction of apoptosis by Ad-REIC, and the other 5 cell lines underwent apoptosis at marginal rates even at 100 MOI (Fig. 1B).

To reveal underlying mechanisms of the difference in the sensitivities of the two groups, we first examined the infection efficiency of an adenovirus. The infection efficiency of Ad-LacZ of KK47, T24, 5637 and UM-UC-3 was high, but J82 and TCCSUP showed lower infection efficiency (Fig. 2). Since our previous studies showed that BiP/Grp78 and the Bcl-2 family proteins are responsible for resistance to Ad-REIC (7,20), we examined the expression levels of heat shock/

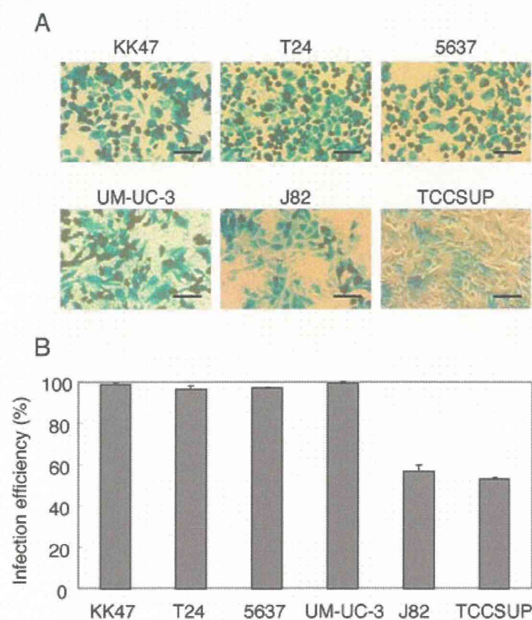

Figure 2. Infection efficiency of adenovirus in bladder cancer cell lines. (A) Cells were infected with an adenovirus vector carrying LacZ (100 MOI). After 48 h, β-galactosidase activity was visualized by staining with X-gal. Bars, 100 μm. (B) Rate of infected cells in bladder cancer cell lines.

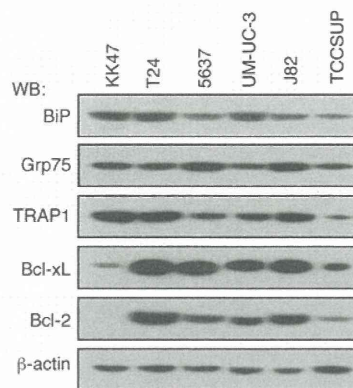

Figure 3. Expression of heat shock/chaperone proteins and Bcl-2 family proteins in bladder cancer cell lines. Western blot analysis (WB) for heat shock/chaperone proteins and Bcl-2 family proteins was performed. β-actin was used as a loading control.

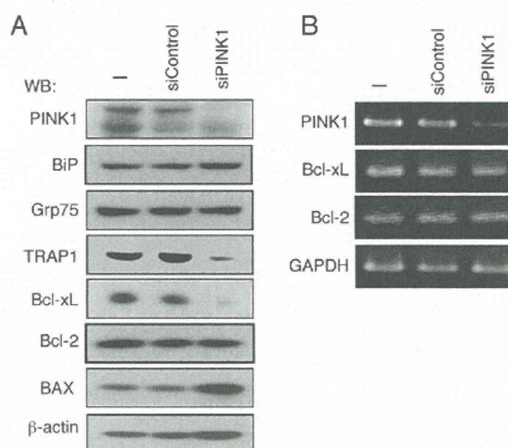

Figure 4. Effect of downregulation of PINK1. (A) Western blot analysis (WB) for heat shock/chaperone proteins and Bcl-2 family proteins was performed 72 h after transfection of siRNA for PINK1. (B) The relative mRNA levels of Bcl-x_L and Bcl-2 were measured by RT-PCR. GAPDH was used as a loading control.

chaperone proteins and Bcl-2 family proteins (Fig. 3). No significant change in the expression of BiP, Grp75 and TRAP1 was observed in the bladder cancer cell lines. On the other hand, Bcl-x_L and Bcl-2 were upregulated in Ad-REIC-resistant cancer cell lines. These results suggest that the resistance of bladder cancer cells to Ad-REIC was partly due to low infection efficiency of Ad-REIC (J82 and TCCSUP) and high expression of mitochondrial anti-apoptotic proteins such as Bcl-x_L and Bcl-2 (T24, 5637, UM-UC-3, J82 and TCCSUP).

Protein expression of Bcl-x_L is reduced by downregulation of PINK1. Since our previous studies showed that PINK1, a mitochondrial protein, could protect cancer cells, we explored the use of PINK1 as a target to overcome the resistance of bladder cancer cells to Ad-REIC-induced apoptosis. Downregulation of endogenous PINK1 by siRNA reduced basal levels of TRAP1 and Bcl-x_L and increased basal level of BAX in T24 cells (Fig. 4A). RT-PCR analysis revealed that mRNA expression level of Bcl-x_L was not changed by downregulation of PINK1 (Fig. 4B).

Downregulation of PINK1 sensitizes bladder cancer cells that are resistant to Ad-REIC-induced apoptosis. We examined the combination effect of downregulation of PINK1 and Ad-REIC for apoptosis induction. We used T24, 5637 and UM-UC-3 cells because these cell lines are resistant to Ad-REIC by high expression of mitochondrial anti-apoptotic proteins but not by low infection efficiency of adenovirus. Overexpression of REIC/Dkk-3 following downregulation of PINK1 induced apoptosis at significantly higher levels in all three cell lines (Fig. 5).

Downregulation of PINK1 augments MG132-induced ROS formation. Cellular level of hydrogen peroxide, a major ROS, was determined by BES-H_2O_2 staining. Hydrogen peroxide produced by stimulation with MG-132 was significantly increased in PINK1-downregulated cells compared with that in untreated cells (Fig. 6).

Discussion

In this study, we showed that downregulation of PINK1 partially sensitized bladder cancer cells to Ad-REIC *in vitro*. As shown in Fig. 1, five bladder cancer cell lines (T24, 5637, UM-UC-3, J82 and TCCSUP) were resistant and only one cell line (KK47) was sensitive to Ad-REIC treatment. Infection efficiency of the adenovirus of two cell lines (J82 and TCCSUP) was limited and this may explain, at least partly, the lower sensitivity to Ad-REIC. A possibility to overcome this obstacle is to create a new version of the adenovirus vector that has higher infection efficiency and potent gene expression capacity. This project has almost been completed in our laboratory.

Our previous study revealed that the Bcl-2 family of mitochondrial proteins was responsible for resistance of bladder

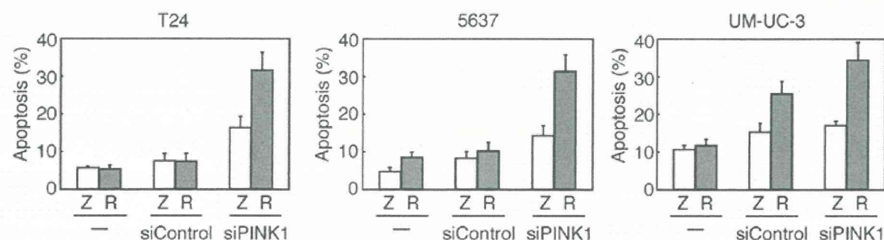

Figure 5. Sensitization of bladder cancer cells to Ad-REIC by downregulation of PINK1. T24, 5637 and UM-UC-3 cells were transfected with indicated siRNAs. Forty-eight hours later, cells were infected with an adenovirus vector (100 MOI) carrying REIC (R) or LacZ (Z), and apoptotic rate of cells was determined after incubating for another 48 h by staining with Hoechst 33342.

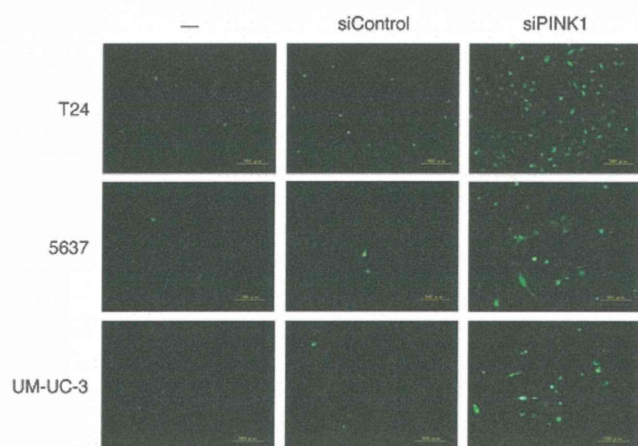

Figure 6. Downregulation of PINK1 augments MG132-induced ROS formation. T24, 5637 and UM-UC-3 cells were transfected with indicated siRNAs 60 h prior to treatment with MG-132 (1 μM, 12 h). BES-H_2O_2 staining (1 μM, 1 h) was performed to visualize hydrogen peroxide. Bars, 500 μm.

cancer cells to Ad-REIC (7). In accordance with this, Bcl-2 family proteins were upregulated in all resistant cell lines (Fig. 3). One possibility to sensitize these cell lines to Ad-REIC is to apply Bcl-2 inhibitors, but our previous study showed that typical Bcl-2 inhibitors were non-selective and showed strong toxicity to normal cells as well as cancer cells (19).

We also reported that resistant clones isolated from sesitive PC3 prostate cancer cells expressed BiP/Grp78, an ER-resisting chaperone protein, at enhanced levels (20). Downregulation of BiP/Grp78 with specific siRNA sensitized the resistant clones to Ad-REIC. Scirrhous gastric cancer cells were shown to be resistant to Ad-REIC and to express BiP/Grp78 at a higher level (data not shown). On the other hand, levels of BiP/Grp78, Grp75 and TRAP1 in the resistant bladder cancer cell lines were not as high as those in scirrhous gastric cancer cells, and no remarkable difference in the expression levels of BiP/Grp78, Grp75 and TRAP1 was noted between the sensitive and resistant bladder cancer cell lines (Fig. 3). These results indicate that the strategy using BiP/Grp78 inhibition is not promising for sensitizing Ad-REIC-resistant cancer cells.

Mitochondria are dynamic organelles that generate energy for cell functions and regulate apoptosis. Recent studies have revealed that interaction of mitochondria and ER contributes to induction of apoptosis (21). For example, apoptosis signaling from the ER is passed to mitochondria. Recently, Iwasawa *et al* revealed that mitochondria transmitted signals to the ER to regulate apoptosis (22). Our previous research demonstrated that Ad-REIC induced apoptosis through ER stress (23). Thus, mitochondria are key organelles through which modulation of cellular sensitivity to Ad-REIC could be developed.

To regulate mitochondria function, we focused on PINK1, a familial Parkinson's disease-linked gene. Downregulation of BRPK/PINK1 has been previously reported to sensitize neuroblastoma cells to various stresses (13). Our hypothetic strategy for sensitizing resistant bladder cancer cells to Ad-REIC was mitochondria dysfunction induced by downregulation of PINK1. Indeed, downregulation of PINK1 could sensitize resistant bladder cancer cells to Ad-REIC (Fig. 5). Furthermore, our preliminary experiments demonstrate that downregulation of PINK1 in resistant bladder cancer cells under similar conditions resulted in sensitization of the cells to oxidative stress induced by MG-132 and enhanced production of ROS (Fig. 6). The results of the present and previous studies (15) indicate that PINK1 can be a new target molecule to sensitize resistant bladder cancer cells to Ad-REIC.

Acknowledgements

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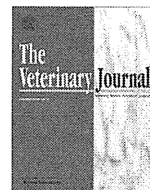

Molecular cloning and tumour suppressor function analysis of canine REIC/Dkk-3 in mammary gland tumours

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ABSTRACT

REIC/Dkk-3, a member of the human Dickkopf (Dkk) family, plays a role as a suppressor of growth in several human cancers. In this study, the tumour suppression function of canine REIC/Dkk-3 was investigated. The full-length open reading frame of the canine REIC/Dkk-3 homologue was cloned and the tissue distribution of REIC/Dkk-3 mRNA was determined, along with the subcellular localisation of the REIC/Dkk-3 protein in canine cancer cell lines. Expression of REIC/Dkk-3 was lower in mammary gland tumours and in canine mammary carcinoma cell lines than in normal mammary gland tissue. Overexpression of REIC/Dkk-3 induced apoptosis in canine mammary carcinoma cell lines. These results show that expression of REIC/Dkk-3 is downregulated in canine mammary tumours and that one of the functions of this gene is induction of apoptosis.

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Introduction

The gene encoding 'Reduced expression in immortalised cells' (*REIC*) is a tumour suppressor gene identical to the Dickkopf-3 (*Dkk-3*) gene (Tsuji et al., 2000). REIC/Dkk-3 is ubiquitously expressed in normal cells in mice and humans, whereas its expression is significantly downregulated in cancer cells (Zhang et al., 2010). We and other investigators have previously shown that overexpression of REIC/Dkk-3 selectively induces apoptosis in multiple cancer cell lines, but not in normal cells (Hsieh et al., 2004; Abarzua et al., 2005; Kashiwakura et al., 2008; Mizobuchi et al., 2008; Sakaguchi et al., 2009).

Endoplasmic reticulum (ER) stress-induced signalling is activated during REIC/Dkk-3-induced apoptosis (Sakaguchi et al., 2009). ER stress plays a role in the induction of apoptosis and occurs when specific glycosylated proteins are overexpressed and protein folding and secretion are impaired (Herr and Debatin, 2001). The GRP78 protein (also called BiP), which is associated with protein folding in the ER and is a key signalling molecule of ER stress (Shen et al., 2002), is upregulated during REIC/Dkk-3-induced apoptosis (Kashiwakura et al., 2008). In addition, induction of caspase-dependent apoptosis by REIC/Dkk-3 is regulated by JNK phosphorylation, along with ER stress (Abarzua et al., 2005; Kashiwakura et al., 2008).

In a previous study, we demonstrated that REIC/Dkk-3 plays a role in monocyte differentiation and tumour regression (Watanabe et al., 2009). Intratumoral administration of REIC/Dkk-3 suppresses tumour growth, resulting in an accumulation of dendritic cells ($CD11c^+$) and cytotoxic T cells ($CD8^+$), and enhanced the anti-cancer activity of splenocytes. Mouse and human studies have shown that REIC/Dkk-3 induces tumour-specific apoptosis and enhances anticancer immunity (Sakaguchi et al., 2009; Watanabe et al., 2009), and its activity is currently being tested in clinical trials in human cancer patients.[1]

A tumour suppressor function of *REIC* has not been investigated in dogs previously. There is only a predicted sequence of the REIC/Dkk-3 homologue in dogs on the ERL database.[2] Although REIC/Dkk-3 is a secreted protein (Tsuji et al., 2000), secreted peptides are not identified within the predicted sequence of canine REIC/

[1] See: http://clinicaltrials.gov/ct2/show/NCT01197209?term=REIC%2FDkk-3&rank=1.
[2] See: http://www.genome.gov/11008069.

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Dkk-3. The elucidation of the structure and tumour suppressor functions of canine REIC/Dkk-3 may help to establish novel therapeutic strategies for treating canine mammary gland tumours.

Mammary tumours are common in female dogs, as well as in women, and there are some molecular and biological similarities between canine and human mammary tumours (Moe, 2001; Egenvall et al., 2005; Rivera and von Euler, 2010; DeSantis et al., 2011). In this study, we investigated the structure and tumour suppressor function of canine REIC Dkk-3. We postulated that, due to its strong tumour suppressor function, REIC/Dkk-3 may be suitable as a new therapeutic agent for canine mammary tumours. We cloned the full-length open reading frame (ORF) of canine REIC/Dkk-3 and investigated the tissue distribution of REIC/Dkk-3 mRNA and the subcellular localisation of the REIC/Dkk-3 protein in canine cancer cell lines. In addition, we examined the expression of REIC/Dkk-3 protein in canine mammary tumours and in normal mammary glands. To confirm the tumour suppressor function of REIC/Dkk-3, we investigated whether the expression of REIC/Dkk-3 in canine mammary tumour cell lines induces apoptosis.

Materials and methods

cDNA cloning and sequencing of canine REIC/Dkk-3

Canine REIC/Dkk-3 was amplified by PCR using the following oligonucleotide primers: cREIC/Dkk-3F (5′-ATGCGGCGGCTCGGGGGCACCCTGCTGTGC-3′) and cREIC/Dkk-3R primer (5′-CTAAATCTCTCCTCCTTCCAGCA-3′). The primers were designed from the sequences of the canine EST database[3] (GenBank DN376871.1) and the predicted sequence of canine REIC/Dkk-3 homologue (GenBank XM_534060.2).

RNA was obtained from canine total brain RNA (Biochain) and reverse transcribed using SuperScript III (Life Technologies). PCR amplification was performed using PrimeSTAR (Takara) and dATP was added to the PCR products using a 10× A-attachment kit (Toyobo). PCR products were cloned into pGEM-T Easy (Promega). The sequences were determined for at least five independent clones (ABI 3100; Applied Biosystems). Nucleotide and amino acid (aa) sequences were analysed and compared with the canine predicted sequence and human and mouse sequence with Genetyx software.

Tissue samples

With permission from the Ethics Committee, we obtained tissue samples from the Department of Veterinary Pathology, School of Veterinary Science, Nippon Veterinary and Life Science University (approval number 11-50, date of approval 27 May 2011). The study included three samples from mammary tubulopapillary carcinomas, two from mammary solid carcinomas and three from non-neoplastic mammary gland tissues. The tissue samples for immunoblot analysis were stored at −70 °C, whereas the tissue samples for histopathological examination were fixed in formalin, dehydrated in alcohol, embedded in paraffin wax, sectioned and stained with haematoxylin and eosin. All the samples were classified by veterinary pathologists according to the WHO classification (Misdorp et al., 1999).

Cell lines and culture

Madin-Darby canine kidney cells (MDCK) and 293T cells were purchased from the American Type Culture Collection (ATCC). Six canine mammary carcinoma cell lines (CIP-p, CIP-m, CHM-p, CHM-m, CNM-p and CNM-m) were kindly provided by Dr N. Sasaki, Laboratory of Veterinary Surgery, University of Tokyo, Japan; these cell lines were established from primary mammary tumours of three dogs that also had metastatic lesions (Uyama et al., 2006). The cell lines were maintained in RPMI 1640 (Wako) supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin (Life technologies), and incubated at 37 °C in an atmosphere containing 5% CO_2.

Tissue distribution of REIC/Dkk-3 mRNA

Reverse transcriptase (RT)-PCR reactions were performed using total RNA from ~24 tissues obtained from the Dog Tissue Total RNA Panel (Zyagen). PCR amplification of canine REIC/Dkk-3 was performed using the following primers: 5′-ATGAGACCAACACGGAAACC-3′ and 5′-CTAAATCTCTCCTCCTTCCAGC-3′. Hypoxanthine phosphoribosyltransferase (HPRT) and β-glucuronidase (GUSB) were used as RT-PCR controls (Brinkhof et al., 2006).

Transfections and immunostaining

To generate haemagglutinin (HA)-tag fusion proteins, the *SalI*/*EcoRI* fragment of REIC/Dkk-3 cDNA was cloned into the pMACS Kk.HA (C) vector (Miltenyl Biotech) (Fig. 1a). Immunocytochemical staining for REIC/Dkk-3 in CHM-p cells overexpressing REIC/Dkk-3 was performed by co-staining for the ER using the ER-ID Red Assay Kit (Enzo Life Sciences). Cells were plated and cultured to 30–40% confluency in LabTek chambers (Nalgene) and were transfected with the pMACS Kk.HA (C) vector containing HA-Tagged canine REIC/Dkk-3 by FuGENE HD (Promega). Forty-eight hours after transfection, the cells were fixed with 4% paraformaldehyde in 100 mM phosphate buffer and blocked with 5% normal goat serum in phosphate buffered saline (PBS). The cells were incubated with polyclonal anti-HA antibodies (1:100 dilution) (561, MBL) overnight at 4 °C and then with fluorescein isothiocyanate-conjugated anti-rabbit secondary antibody (Molecular Probes) for 1 h. To stain the nuclei, the cells were incubated with Hoechst 33342 (Dojindo) for 15 min at room temperature. The fluorescent staining was visualised under a fluorescence microscope system (BZ-9000; Keyence).

Immunoblot analysis

293T cell monolayers were cultured to 80% confluence on 6-well plates and the cells were transfected as described above. The cells were lysed in ice-cold radioimmunoprecipitation (RIPA) buffer (Nacarai Tesque) and incubated for 15 min at 4 °C. Canine tissue was homogenised in ice-cold RIPA buffer. Insoluble fragments were removed by centrifugation at 16,000 g for 10 min at 4 °C and supernatants were stored at −80 °C. Protein concentrations were determined using a Protein Assay Bicinchoninate kit (Nacarai). Extracted protein (~10 µg) was mixed with 6x loading buffer, consisting of 450 mM Tris pH 6.8, 45% sucrose, 5% β-mercaptoethanol, 15% sodium dodecyl sulphate (SDS) and bromophenol blue, and separated by electrophoresis on 12% SDS-polyacrylamide gel electrophoresis gels (Bio-Rad). Proteins were electro-transferred onto polyvinylidene fluoride membranes in 25 mM Tris, 192 mM glycine and 20% methanol. Following transfer, membranes were blocked with 10% non-fat dry milk and 6% glycine in Tris-buffered saline containing 0.1% Tween-20 and were developed with rabbit polyclonal anti-human REIC/Dkk-3 antibody (10365-1-AP, Proteintech) and antibodies specific for caspase-3 (9962, Cell Signaling Technology), cleaved-caspase-3 (9961, Cell Signaling Technology) and β-actin (sc-69879, Santa Cruz). Horseradish peroxidase-conjugated secondary antibodies and the electrochemiluminescence (ECL) Pro substrate kit (Perkin Elmer) were used for the detection of antibody-bound proteins. Densitometric analysis was performed and the REIC/Dkk-3-to-β-actin ratio was calculated using Image J software (version 1.44).[4]

Apoptosis assay

To examine the in vitro induction of apoptosis after treatment, CIP-p cells were seeded in flat-bottomed 6-well plates and incubated for 24 h. Cells were then treated with the control adenovirus (Ad), Ad-LacZ or Ad-human (h) REIC/Dkk-3 at the indicated multiplicity of infection (MOI) in serum-free medium (500 µL) for 2 h, then the medium was exchanged with fresh complete medium (2 mL). After further incubation for 72 h, Hoechst 33342 stock solution was added to the medium to a final concentration of 2 µg/mL and the cells were incubated in the dark for 10 min (Kawasaki et al., 2009). Apoptotic cells were identified on the basis of the presence of highly condensed or fragmented nuclei using fluorescence microscopy. Apoptotic cells were counted in five different fields under a microscope; 100 cells were evaluated in each field.

Statistical analysis

Student's *t* test was used to compare the difference between the mean values. P values <0.05 were considered to be statistically significant.

Results

Cloning and structural analysis of canine REIC/Dkk-3

The ORF of canine REIC/Dkk-3 cDNA determined in this study (GenBank AB733648) had 1047 base pairs (bp) and was predicted to code for 348 aas. The full length ORF of canine REIC/Dkk-3 was 213 bp and 71 aas longer than the predicted sequence of canine REIC/Dkk-3 (GenBank XM_534060.2, ORF: 834 bp, 277 aas). This discrepancy is due to a missense mutation accompanied by a stop codon in XM_534060.2 (positions 200-202), which is located between the first and second ATGs of AB733648 (Fig. 1a). Therefore,

[3] See: http://www.ncbi.nlm.nih.gov/dbEST/index.html.

[4] See: http://rsb.info.nih.gov/ij.

a

cREIC/Dkk-3F
1st ATG 2nd ATG
DN376871.1
cREIC/Dkk-3R
-101 TAG TAG
XM_534060.2
-30 Primers for RT-PCR
AB733648
1 1048
SalI Primers for SalI/EcoRI fragment of REIC/Dkk-3 EcoRI
CMV promoter HA-Tag
pMACS Kk.HA (C)

b

```
Canine     1 MRRLGGTLLCLLLAAAVPTAPAPAPTAPPAPAEAGPALSYPQEEATLNEMFREVEELMED
Human      1 MQRLGATLLCLLLAAAVPTAPAPAPTATSAPVKPGPALSYPQEEATLNEMFREVEELMED
Mouse      1 MQRLGGILLCTLLAAAVPTAPAPSPTVTWTPAEPGPALNYPQEEATLNEMFREVEELMED
             *:***. *** ************:**.. :*.:.****.********************

Canine    61 TQHKLRSAVEEMEAEEAAAKTS-EVNLANLPSSYHNETNTETRVGNNTIHVHREIHKVTN
Human     61 TQHKLRSAVEEMEAEEAAAKASSEVNLANLPPSYHNETNTDTKVGNNTIHVHREIHKITN
Mouse     61 TQHKLRSAVEEMEAEEAAAKTSSEVNLASLPPNYHNETSTETRVGNNTVHVHQEVHKITN
             *******************:* *****.**..*****.*:*:*****:***:*:**:**

Canine   120 NQTGRTVFSETVITSVGDEEGKKSHECIIDEDCGPTRYCQFASFEYTCQSCRDQQTLCTR
Human    121 NQTGQMVFSETVITSVGDEEGRRSHECIIDEDCGPSMYCQFASFQYTCQPCRGQRMLCTR
Mouse    121 NQSGQVVFSETVITSVGDEEGKRSHECIIDEDCGPTRYCQFSSFKYTCQPCRDQQMLCTR
             **:*: ***************::************: ****:**:****.**.*: ****

Canine   180 DGECCGDQLCVWGHCAKTATRGGNGTICDNQRDCQPGLCCAFQRGLLFPVCTPLPVEGEL
Human    181 DSECCGDQLCVWGHCTKMATRGSNGTICDNQRDCQPGLCCAFQRGLLFPVCTPLPVEGEL
Mouse    181 DSECCGDQLCAWGHCTQKATKGGNGTICDNQRDCQPGLCCAFQRGLLFPVCTPLPVEGEL
             *.********.****:: **:*.*************************************

Canine   240 CHDPASRLLDLITWELEPDGALDRCPCASGLLCQPHSHSLVYVCKPAFMGSRGEDGESPV
Human    241 CHDPASRLLDLITWELEPDGALDRCPCASGLLCQPHSHSLVYVCKPTFVGSRDQDGEILL
Mouse    241 CHDPTSQLLDLITWELEPEGALDRCPCASGLLCQPHSHSLVYMCKPAFVGSHDHSEESQL
             ****:*:***********:***********************:***:*:**:...  *   :

Canine   300 PREAPSEYEDGSFIEEVRQELENLERSLSVEMALGEPDAPSELLEGGE-I 348
Human    301 PREVPDEYEVGSFMEEVRQELEDLERSLTEEMALREPAAAAAALLGGEEI 350
Mouse    301 PREAPDEYEDVGFIGEVRQELEDLERSLAQEMAFEGP-APVESLGGEEEI 349
             ***.*.***  .*: *******:*****: ***:  * *    * * * *
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Fig. 1. Cloning strategy and sequence alignments of canine REIC/Dkk-3. (a) Alignment of nucleic acid sequence identified in this study (AB73348) with the expressed sequence tag (EST) sequence (DN376871.1) and the predicted canine REIC/Dkk-3 sequence (GenBank XM_534060.2). The identical sequences are indicated in black boxes. The putative start codons (1st and 2nd) and the stop codon are indicated with dotted line. The sequence that was different from the sequence identified in this study and in the EST database, including the stop codon, is indicated with a grey box. The primers that were used for cloning and sequencing (cREIC/Dkk-3F and R) and subcloning into the pMACS Kk.HA (C) vector (the primers were attached to *Sal*I or *EcoR*I site) to generate the haemagglutinin (HA)-tagged fusion protein and for RT-PCR examination are indicated by arrows. (b) Comparison of the amino acid (aa) sequence of canine REIC/Dkk-3 identified in this study (AB733648) with human REIC/Dkk-3 (NP_056965) and mouse REIC/Dkk-3 (NP_056629). The predicted aa sequence was aligned using ClustalW. '* —' Single fully conserved aa. ': —' Conservation of the strong amino group. '. —' Conservation of the weak amino group. The signal peptide sequence that was predicted using the SignalP software is underlined. The N-terminal containing 78 aas that induced apoptosis in human prostate cancer cell lines is in a box. The potential binding site of human dynein light chain, Tctex-1 is indicated in boldface.

the position of the start codon of XM_534060.2 corresponds to the second ATG of AB733648 (Fig. 1a). A putative secretion signal sequence was present in the sequence we identified, suggesting that canine REIC/Dkk-3 is a secreted protein (Fig. 1b). The canine REIC/Dkk-3 Protein shows 85% and 82% homology with the human and murine REIC/Dkk-3 proteins, respectively. Canine REIC/Dkk-3 includes a putative secretion signal sequence and an aa sequence motif [E-X-G-R/K-R/K-X-H], previously identified as the Tctex-1-binding domain of dynein intermediate chain (Fig. 1b) (Ochiai et al., 2011). Canine REIC/Dkk-3 has a well-conserved stretch of 78 aas at the N-terminus, which is sufficient to induce apoptosis in human prostate cancer cell lines (Abarzua et al., 2008). Therefore, we hypothesised that canine REIC/Dkk-3 can induce apoptosis in cancer cells.

Tissue distribution and cell localisation of canine REIC/Dkk-3

The tissue distribution of canine REIC/Dkk-3 mRNA was investigated by RT-PCR in 24 normal canine tissues. As previously reported in humans (Tsuji et al., 2000), REIC/Dkk-3 mRNA was

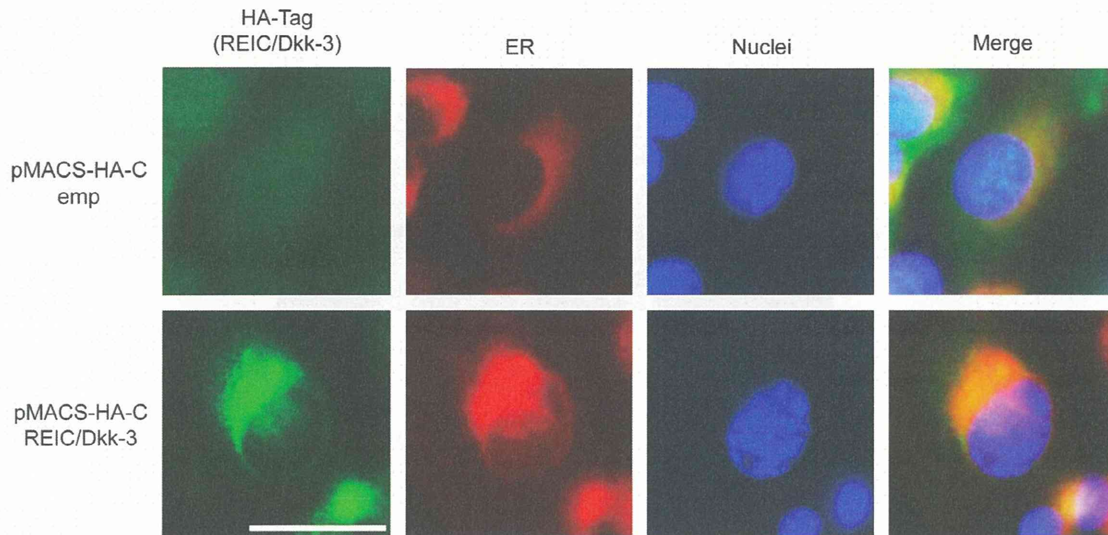

Fig. 2. Subcellular localisation of canine REIC/Dkk-3. Co-localisation of REIC/Dkk-3 and endoplasmic reticulum (ER) was examined by double immunofluorescence staining and imaged using fluorescence microscopy. Images in green, red and blue show the subcellular localisation of the haemagglutinin (HA) tag alone, or HA-tagged REIC/Dkk-3, ER and nuclei, respectively. The areas of overlap between the REIC/Dkk-3 and ER are shown in yellow in the overlay image. The image merging was performed using BZ-Analyzer software (Keyence). Bar = 50 μm.

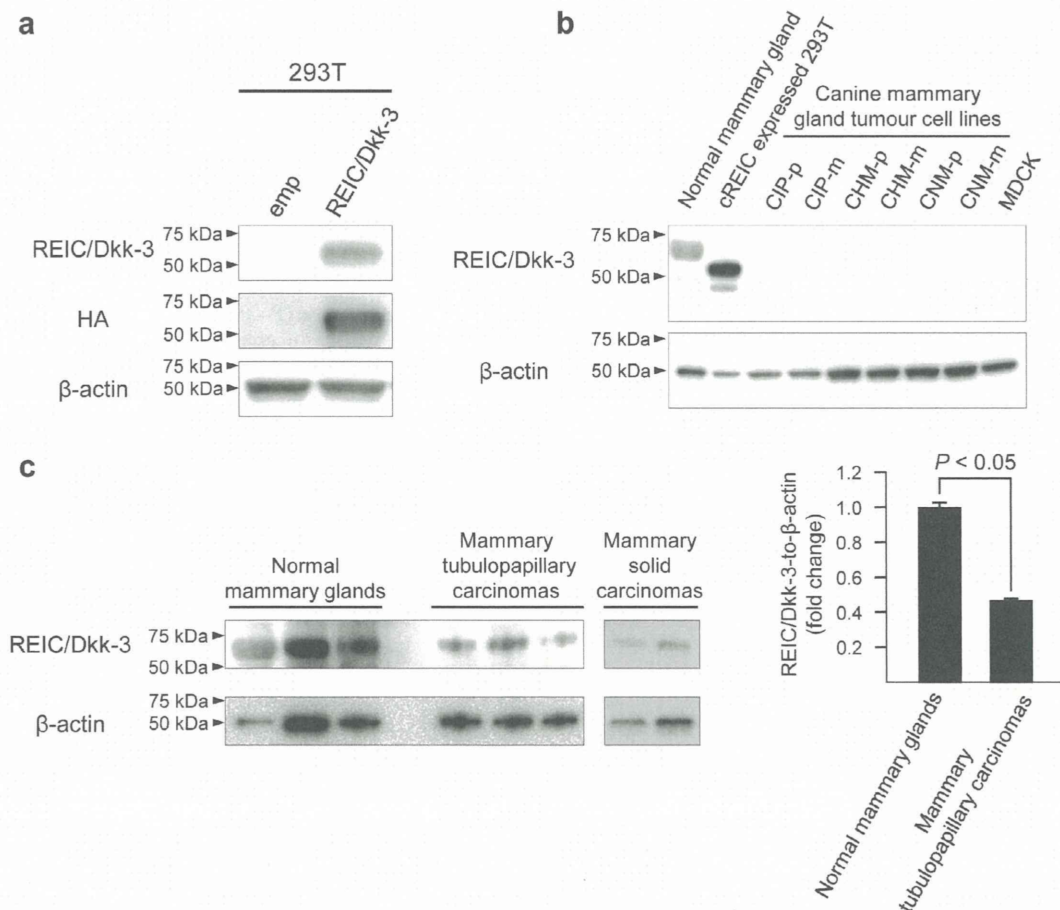

Fig. 3. Expression of REIC/Dkk-3 protein in canine cell lines and mammary gland tumours. (a) To test the cross-reactivity of anti REIC/Dkk-3 antibodies for the canine protein, immunoblotting was performed in 293T cells that were transfected with an empty haemagglutinin (HA)-tagged vector alone (emp) or HA-tagged canine REIC/Dkk-3. β-Actin was used as a reference protein for normalisation for protein loading. (b) Expression levels of canine REIC/Dkk-3 protein were determined by immunoblot analysis of normal canine mammary glands and canine mammary gland cancer-derived cell lines. β-actin was used as a loading control. (c) Protein levels of REIC/Dkk-3 were analysed in three normal mammary glands (controls), three tubulopapillary mammary carcinomas and two solid mammary carcinomas by Western blot analysis. The results shown are representative of three independent experiments. All immunoblots were performed on independent membranes, although reprobing was not performed.

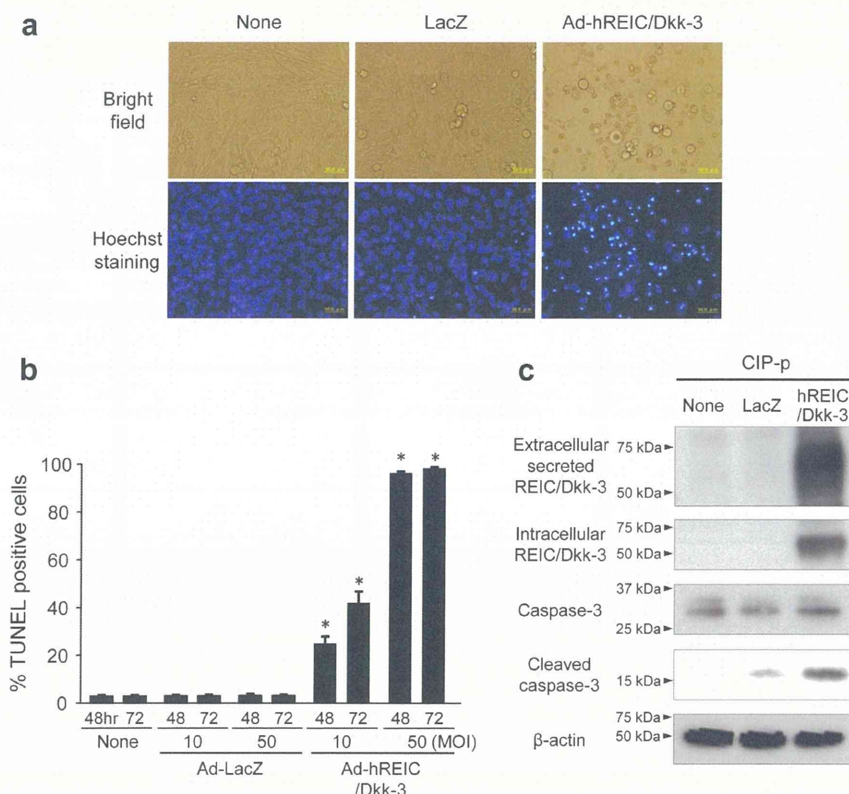

Fig. 4. Induction of apoptosis in canine mammary gland tumour cell lines due to overexpression of REIC/Dkk-3. (a) Induction of apoptosis in CIP-p cells after Ad-hREIC/Dkk-3 treatment was examined using Hoechst 33342 staining. Cells were infected with 50 multiplicities of infection (MOIs) of Ad-LacZ, Ad-REIC/Dkk-3 or control vector alone and incubated for 72 h. (b) Percentages of apoptotic CIP-p cells at 48 and 72 h after treatment with Ad-hREIC/Dkk-3 at different MOIs. A total of five different fields were examined under a microscope to determine the apoptotic rate. A statistically significant difference was observed between Ad-hREIC/Dkk-3 and the control Ad-LacZ treatment (* $P < 0.05$). (c) Western blot analysis for the indicated proteins in CIP-p cells treated with Ad-REIC/Dkk-3. The extracellularly secreted REIC/Dkk-3 was detected in the culture supernatant 48 h after transfection. Bar = 50 μm.

ubiquitously expressed in all canine tissues that were examined (see Appendix A: Supplementary Fig. 1). The REIC/Dkk-3 protein exhibited a punctate localisation pattern in the cytoplasm when examined by immunostaining (Sakaguchi et al., 2009); our previous studies showed that the REIC/Dkk-3 protein is predominantly localised to the ER in human and murine cells (Zhang et al., 2010; Ochiai et al., 2011). We therefore performed co-staining experiments for ER and REIC/Dkk-3 in a canine cell line which was transiently transfected with HA-tagged canine REIC/Dkk-3. REIC/Dkk-3 was mainly localised around the ER in canine cells (Fig. 2).

Expression of REIC/Dkk-3 protein in canine mammary gland tumour cell lines and tissues

Since antibodies recognising canine REIC/Dkk-3 are not available, we first performed immunoblotting in 293T cells transfected with canine REIC Dkk-3 using antibodies that detect the HA-tagged canine REIC/Dkk-3 protein (Fig. 3a). Bands of the same size were detected with anti-HA antibody and anti-human REIC-Dkk-3 antibodies, confirming that the antibodies against human-REIC/Dkk-3 can detect canine REIC/Dkk-3. Next, we examined REIC/Dkk-3 expression in normal canine mammary glands and in various cell lines derived from canine mammary gland tumours. In the normal canine mammary gland, the REIC/Dkk-3 protein was detected as a single band ~60 kDa by immunoblot analysis. In contrast, REIC/Dkk-3 protein was barely detectable in six canine mammary grand cancer cell lines, nor in MDCK cells (Fig. 3b). Consistent with this observation, the levels of canine REIC/Dkk-3 protein were lower in canine mammary tubulopapillary carcinomas than in normal mammary gland tissue ($P < 0.05$) (Fig. 3c). A solid mammary carcinoma also showed reduced expression of REIC/Dkk-3.

Apoptosis of canine mammary gland tumour cell lines induced by overexpression of REIC/Dkk-3

To examine a possible use of REIC/Dkk-3 as a tool for targeted gene-based therapy for canine mammary gland tumours, we overexpressed REIC/Dkk-3 in cancer cells by using a replication-deficient adenovirus vector. Within a few days after infection, most of the tumour cells had detached from the bottom of the culture vessels (Fig. 4a). Hoechst 33342 staining confirmed that apoptotic cells were frequently observed in Ad-hREIC/Dkk-3-treated CIP-p cells. The extent of apoptosis 72 h after treatment at MOIs of 10 and 50 was 42.0% and 98.6%, respectively. Significant induction of apoptosis was observed in Ad-hREIC/Dkk-3-treated cells, unlike that observed in control cells treated with Ad-LacZ ($P < 0.05$) (Fig. 4b). These results confirmed that human REIC/Dkk-3 overexpression induced apoptosis in canine mammary gland cancer cells, suggesting that Ad-hREIC/Dkk3 may be useful as a new therapeutic agent for canine tumours.

Next, we determined whether REIC/Dkk-3-induced apoptosis in CIP-p cells was caspase-dependent. The intra- and extracellular levels of REIC/Dkk-3 protein increased in transfected CIP-p cells, but not in cells infected with the Ad-LacZ vector alone (Fig. 4c). Infection of CIP-p cells with Ad-REIC/Dkk-3 did not alter the levels of caspase-3, but increased the expression of cleaved caspase-3 (Fig. 4c).

Discussion

In this study, we cloned and sequenced the full-length ORF of canine REIC/Dkk-3. Although a putative sequence was predicted by automated computational analysis from a genomic sequence, no information was available concerning its gene. Since human and murine REIC/Dkk-3 are recognised as strong tumour suppressor genes, we cloned the entire sequence of canine REIC/Dkk-3 to investigate the function of this protein in dogs. The full-length canine REIC/Dkk-3 ORF was amplified by RT-PCR; the expressed protein was 71 aas longer than the predicted sequence and our sequence had a putative signal peptide in the 5′ region.

In our previous study, we showed that REIC/Dkk-3 interacts with human dynein light chain, Tctex-1, at aa sequence motif [E-X-G-R/K-R/K-X-H] (Ochiai et al., 2011). Since canine REIC/Dkk-3 has a well-conserved Tctex-1-binding motif, the interaction of REIC/Dkk-3 and Tctex-1 is likely to be conserved across all mammalian species. Canine REIC/Dkk-3 has a well-conserved segment of 78 aa at the N-terminus, which induces apoptosis in human prostate cancer cell lines (Abarzua et al., 2008), suggesting that canine REIC/Dkk-3 also has a tumour suppressor activity.

RT-PCR analysis demonstrated that REIC/Dkk-3 is expressed in a variety of canine organs. Although previous reports have shown that REIC/Dkk-3 mRNA and protein are expressed at very low levels in the spleen and in peripheral blood leucocytes (Tsuji et al., 2000; Zhang et al., 2010), we observed REIC/Dkk-3 expression in all 24 canine organs examined. We showed that expression of REIC/Dkk-3 protein is downregulated in canine mammary gland cancer cell lines and in mammary gland tumours. It has been reported that human REIC/Dkk-3 gene expression is reduced by promoter-hypermethylation (Kobayashi et al., 2002); whether REIC/Dkk-3 is epigenetically silenced in canine tumours remains to be determined.

Increased expression of REIC/Dkk-3 in CIP-p cells led to an increase in the number of apoptotic cells. Consistent with this observation, caspase-3 was activated by REIC/Dkk-3. Therefore, the anti-proliferative property of REIC/Dkk-3 may be due to induction of caspase-dependent apoptosis. Our findings indicate that REIC/Dkk-3 regulates caspase-dependent apoptosis in canine mammary gland tumour cell lines. Mouse and human studies have shown that forced expression of hREIC/Dkk-3 enhances anticancer immunity (Sakaguchi et al., 2009; Watanabe et al., 2009). The role of REIC/Dkk3 in anticancer immunity in canine mammary gland tumours requires further investigation.

In this study, we used human REIC/Dkk-3 in an adenoviral vector. This construct is used in ongoing clinical trials in human prostate cancer patients and its safety and toxicity in dogs was tested following Good Laboratory Practice (GLP) toxicology study guidelines before human clinical trials commenced.[5] There is potential for clinical trials using REIC/Dkk-3 as a cancer therapeutic agent for canine mammary gland tumours.

Conclusions

Expression of RECI/Dkk3 is reduced in mammary gland tumours compared to normal mammary glands in dogs. Expression of RECI/Dkk3 induces apoptosis in canine mammary gland tumour cell lines. REIC/Dkk-3 may play a role in regulating cell survival in canine mammary gland tumours by promoting caspase-dependent apoptosis. Inhibition of REIC/Dkk-3 appears to be a critical event in tumour development and progression. This suggests that REIC/Dkk-3 is a promising candidate for targeted molecular therapy in dogs with mammary gland tumours.

[5] See: http://clinicaltrials.gov/ct2/show/NCT01197209?term=REIC%2FDkk-3&rank=1.

Conflict of interest statement

None of the authors have any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tvjl.2013.04.024.

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