The Wnt pathway regulator DKK1 is preferentially expressed in hormone-resistant breast tumours and in some common cancer types

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In addition to new tumour antigens, new prognostic and diagnostic markers are needed for common cancers. In this study, we report the expression of Dickkopf-1 (DKK1) in multiple common cancers. This constitutes a comprehensive analysis of the DKK1 expression profile. Dickkopf-1 expression was evaluated by classical and quantitative reverse transcriptase–polymerase chain reaction (RT–PCR) and enzyme-linked immunosorbant assay for protein determination, in cancer lines and clinical specimens of several cancer origins. For breast cancer, expression was correlated with clinicopathological parameters. Dickkopf-1 expression was confirmed in several cancer cell lines derived from breast and other common cancers. Dickkopf-1 protein secretion was documented in breast, prostate and lung cancer lines, but was negligible in melanoma. Analysis of DKK1 expression in human cancer specimens revealed DKK1 expression in breast (21 out of 73), lung (11 out of 23) and kidney cancers (six out of 20). Interestingly, DKK1 was preferentially expressed in oestrogen and progesterone receptor-negative tumours (ER⁻/PR⁻; P = 0.005) and in tumours from women with a family history of breast cancer (P = 0.024). Importantly, DKK1 protein production was confirmed in multiple breast cancer specimens that were positive by RT–PCR. This work establishes DKK1 as a potential prognostic and diagnostic marker for cohorts of breast cancer patients with poor prognosis. Dickkopf-1 may also become a relevant candidate target for immunotherapy of different cancers.

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MATERIALS AND METHODS

Bioinformatic tools for differential gene expression in breast cancer compared to normal tissues

To find candidate genes, we exploited the Digital Gene Expression Displayer (DGED), a bioinformatic tool freely available from the Cancer Genome Anatomy Project server (http://www.ncbi.nlm.nih.gov/ncigca/p) (Strausberg et al, 2000), probing two different complementary DNA (cDNA) expression libraries, expressed sequence tag (EST; http://cgap.ncbi.nlm.nih.gov/Tissues/GXS) and serial analysis of gene expression (SAGE; http://cgap.ncbi.nlm.nih.gov/SAGE/SDGEDMETHOD = SS10,LS60&ORG = Hs.). These bioinformatic tools allowed the analysis of expression profiles from the EST and SAGE databases by the clustering of libraries by origin. All the available libraries prepared from normal tissues were clustered in one group, and all available libraries prepared from breast cancers were clustered in a second distinct group. Candidate genes were selected on the basis of high expression levels in available human breast cancer libraries, and absent or low levels in normal human tissues from important organs.

Cell culture

The breast cancer cell lines MCF-7, MDA231, BT-20, HCC1428 and HCC2218 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). B lymphocytes immortalized with Epstein–Barr virus (EBV) HCC1428 and HCC2218, as well as the lung cancer lines Calu6, H1299, A549, H460 and H596, the prostate cancer lines DU145, PC-3 and LNCaP, and the kidney cancer line RCC-W were all established at the National Cancer Institute/National Institute of Health, and the SK23 line was acquired from the ATCC. The melanoma lines 397mel, 537mel, 586mel, 888mel, 1087mel, 1088mel, 1200mel, 1337mel and MelS-1 were selected on the basis of high expression levels in available human breast cancer libraries, and absent or low levels in normal human tissues from important organs.

Peripheral blood mononuclear cells (PBMC) were collected from healthy donors recruited by Dr Jean-Pierre Routy at the McGill University Health Centre (MUHC, Royal Victoria Hospital, Montreal, Quebec, Canada). The PBMC were prepared from blood by centrifugation on a lymphocyte separation medium (Cellgro, Herndon, VA and Wisent). To generate CD40-activated B cell lines, and corresponding EBV-B lines, 10 µM HEPES solution (Invitrogen), and 250 µg/ml of phytohemagglutinin (PHA; Sigma, Oakville Ontario, Canada).

Clinical specimens

Breast cancer specimens of 1.5 cm or higher were provided by the Fonds de la recherche en santé du Québec (FRSQ) Cancer Network breast tissue library of CHUM – Hôpital Notre-Dame and Hôpital Hôtel-Dieu (specimens stabilised in RNALater™, Sigma; for reverse transcriptase–polymerase chain reaction (RT–PCR) analyses). Lung cancer specimens were obtained after resection in the Thoracic Surgery Department of CHUM – Hôpital Notre-Dame (five samples; stabilised in RNAlater™), and all others were acquired from the Lung Cancer Tissue Library of the FRSQ Respiratory Health Network of Hôpital Laval (Québec, Québec, Canada; snap-frozen tumour pieces). Kidney cancer specimens were collected after partial or total kidney resection at the Montreal General Hospital (MUHC).

Snap-frozen and RNALater-stabilised cancer samples were homogenised with a Medimachine™ (Dakk Cytomation, Glostrup, Denmark) according to the manufacturer's instructions. RNA was prepared with Qiagen reagent (QIAGEN GmbH, Hilden, Germany), followed by a cleanup and concentration procedure using the RNeasy™ Mini or Micro Kit (QIAGEN) according to the manufacturer's instructions.

The status of the oestrogen and progesterone receptors in the breast cancer specimens was determined by the clinical pathology services of CHUM – Hôpital Notre-Dame and Hôpital Hôtel-Dieu.

Reverse transcriptase–polymerase chain reaction

RNA from cell lines and lymphocytes was prepared with RNeasy™ Mini or Micro Kits (QIAGEN) according to the manufacturer's instructions. Intron-spanning PCR primers were designed from genes selected by the bioinformatic approach. To perform classical and quantitative RT–PCR analyses, cDNA was first synthesised from mRNA (1 µg) with oligo-dt (Invitrogen) using an Omniscript Reverse Transcription Kit (QIAGEN). Classical RT–PCR amplification was undertaken with the HotStartTaq DNA Polymerase (QIAGEN). The cycling conditions were 15 min at 95°C (β-actin) or 32 (DKK1 and other genes) cycles of 45 s at 94°C, 45 s at 55°C, 1 min at 72°C, with a final extension of 10 min at 72°C, in a T3 Thermocycler™ system (Biometra, Göttingen, Germany). The primer sequences for β-actin were: 5’- GGAAGGCTGGAAGAG GTCG; and 3’- GTGATGGTGCGATGGTGC, resulting in a 700-bp amplicon. Amplification was detected by ethidium bromide staining after electrophoresis migration in agarose gel (1.5 or 2%; with apparatus from Bio-Rad, Hercules, CA, USA). The primer sequences were as follows: DKK1 (5’ primer: ATTCACAAGCTTATCAAA GCC; 3’ primer: CCAAGG TGCTATGATCATACC, amplicon 383 bp). For quantitative real-time RT–PCR amplification, was performed in a LightCycler™ system (Roche, Mannheim, Germany) and revealed with an SYBR Green™ Kit (Quantitect™ SYBR Green PCR, QIAGEN). Standard curves for each gene were established to quantify the number of copies for each sample, and expression was considered only when the sample Ct was within the limit of each standard curve. The cycling conditions were 15 min at 95°C, 40 cycles of 15 s at 94°C, 30 s at 55°C, 30 s at 72°C and 5 s at 82°C (β-actin) or 84°C (DKK1). The primer sequences for real-time PCR were β-actin 5’- AAGGCAACACCCGGAG; 3’- TAATGTACGCAAG TTCCGG; DKK1 5’- CTGGTTTCTCAATCCACGG; 3’- GCACCTCT CGTCTCCTCTG. Finally, amplification of the relevant amplicon was confirmed by separation on agarose (2%) gel, revealed as mentioned earlier. β-actin was exploited as a housekeeping gene. A sample was considered positive for DKK1 when amplification was > 200 copies of DKK1 per 10^6 copies of β-actin. This threshold
value was established considering that the expression levels in normal tissues were less than 200 copies, with the exception of the placenta.

**Enzyme-linked immunosorbent assay**

Cancer cell lines and lymphocytes culture medium were tested for the presence of secreted DKK1 protein by enzyme-linked immunosorbent assay (ELISA). Cells were seeded at 1 x 10^5 cells well⁻¹ in flat-bottom 96-well plates (Corning Inc., Corning, NY, USA). Supernatants were harvested after 24 h and assayed for DKK1 with the commercial DuoSet Human DKK1 ELISA Kit, as recommended by the manufacturer (R&D Systems, Minneapolis, MN, USA). The lowest standard point for the ELISA assay was 62 pg ml⁻¹.

Dickkopf-1 protein production was also quantified in breast cancer lysates. Freshly resected breast cancer specimens provided by the FRSQ Cancer Network (as mentioned earlier) were mechanically homogenised in complete AIM-V medium with the Medimachine (Dako Cytomation) to obtain a single-cell suspension. Lysates were prepared by five rapid subsequent freeze/thaw cycles (Lapointe et al., 2003). Cell debris were sedimented, and supernatants were assayed for DKK1 as described above. Cell line lysates used as controls were prepared by the same technique, from 1 x 10^6 cells ml⁻¹ suspension.

**Statistical analyses**

Mean values of DKK1 expression were compared by clinico-pathological clusters with the two-tailed t-test for independent samples. Dickkopf-1-positive tumours were compared to DKK1-negative tumours for the same clusters, using the two-sided Pearson χ² test. Differences were considered significant at P<0.05. Statistics were performed with SPSS 13.0 software for Windows (LEAD Technologies, Chicago, IL, USA).

**RESULTS**

**DKK1 expression in breast cancer cell lines**

We originally intended to find new TA for potential applications in immunotherapy, by exploiting the DGED. This bioinformatic approach was used as a screening tool to highlight potential overexpressed genes in breast cancer. This list of genes predicted by these banks (Supplementary data; Supplementary Table 1) was considered as preliminary data needing to be further investigated. By using these mining tools, some genes predicted to be practically absent from normal tissues later showed expression in various normal tissues by RT–PCR approaches. Dickkopf-1 was found exclusively in the placenta (Figure 2A) as classical and quantitative real-time RT–PCR approaches were not performed with the specific primers indicated. Normal primary cell lines were prepared by stimulation of PBMC with anti-CD3 and IL-2 (T cells), or with soluble CD40L and IL-4, which stimulate B lymphocytes to proliferate (B cells). Reverse transcriptase was omitted in the MDA231-RT group and DKK1 secretion was determined by ELISA. The results presented are representative of at least three independent experiments. Dickkopf-1 was further confirmed in a second cDNA panel (Figure 2B). Cell debris were sedimented, and supernatants harvested after 24 h in 96-well plates as described in Materials and Methods. Supernatants harvested, and RT–PCR analyses were performed with the specific primers indicated. Normal primary cell lines were prepared by stimulation of PBMC with anti-CD3 and IL-2 (T cells), or with soluble CD40L and IL-4, which stimulate B lymphocytes to proliferate (B cells). Reverse transcriptase was omitted in the MDA231-RT group and DKK1 secretion was determined by ELISA. The results presented are representative of at least three independent experiments.

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In summary, we have validated the expression, in breast cancer lines, of a gene previously selected by the bio-informatic approach. Although TA are frequently expressed in both fresh tumour samples and tumour cell lines, confirmation of expression in fresh breast cancer specimens is essential. Also, limited expression in normal tissues needed to be evaluated.

**DKK1 is expressed in the placenta**

Classical and quantitative real-time RT–PCR approaches were next adopted to evaluate DKK1 expression in normal tissues. Dickkopf-1 was found exclusively in the placenta (Figure 2A) as reported previously (Fedi et al., 1999). His restricted expression to the placenta was further confirmed in a second cDNA panel prepared from normal tissues (Figure 2B). We also exploited quantitative real-time RT–PCR to further validate this observation (Figure 2C). Critically, we confirmed DKK1 expression in the
placenta with two distinct, commercially available mRNAs prepared from normal tissues (Origene, Rockville, MD, USA and BD-Clontech, Mountain View, CA, USA), and a weak detection in some normal tissues. Minimal DKK1 expression was further confirmed from normal breast tissues prepared from five different donors (Figure 2D).

**Figure 2**  Expression profile of DKK1 in normal tissues. Messenger RNA was prepared from normal tissues (acquired from Origene in (A), and from BD-Clontech in (B–D) and controls. (A and B) Semiquantitative classical RT–PCR analyses were performed with β-actin and primers indicated as described in Materials and Methods. (C) Expression from normal tissues was evaluated by quantitative real-time RT–PCR. (D) Expression in normal breast tissues was also evaluated by quantitative real-time RT–PCR. The results presented are representative of at least two independent experiments.

**Preferential expression of DKK1 in hormone-resistant breast cancer, in familial cases and in primary tumours from patients with invaded axillary nodes**

To further characterise DKK1 expression in breast cancer tumours, we next assessed DKK1 expression profiles in clinical breast cancer...
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650 specimens. Globally, DKK1 was detected in 21 out of 73 patient specimens evaluated (Figure 3A). The expression profile was clustered based on clinicopathological parameters characterising breast cancer. Dickkopf-1 expression profile was first analysed according to hormone receptors status (oestrogen and progesterone receptors; Figure 3A). Precisely, in DKK1+ tumours, the mRNA level was significantly higher in ER+/PR− compared to other tumours (respectively 818 vs 213 DKK1 mRNA copies/10⁵ β-actin copies, P = 0.009; Figure 3B). Also, as shown in Figure 3C, a statistical difference was demonstrated in the preferential DKK1 expression of hormone-independent tumours (P = 0.005). This preferential expression was observed mainly in the absence of both hormone receptors. Specifically, only one out of the five ER−/PR+ tumours evaluated was DKK1+ (data not shown). Similar results were obtained with the ER+/PR− group (one DKK1+ specimen of eight tested; data not shown).

We also evaluated expression depending on familial history, and 48% of DKK1+ tumours arose from women reporting familial cases of breast cancer (Figure 3D). When we compared the cohort tested, DKK1 expression was preferentially and significantly expressed in women with familial cases of breast cancer (P = 0.024). Dickkopf-1 was also preferentially detected in primary breast cancer tumours.

Figure 3 Dickkopf-1 expression from breast cancer clinical samples. (A) Complementary DNA was prepared from the indicated controls and clinical samples. Amplification was undertaken by real-time PCR and revealed by SYBR green staining. Amplification of the relevant amplicon was further confirmed by separation on agarose gel and ethidium bromide staining. (B and C) Samples from patients are clustered by ER and PR status, as evaluated by the local pathology clinical department (score: −: negative, +: positive, w: weakly positive, NA: not available). Dickkopf-1 levels from mRNA-positive tumours were clustered according to ER/PR status; statistical significance was evaluated by the χ² test. (D) DKK1+ and DKK1− samples were clustered according to the reported familial history of breast cancer; statistical significance was evaluated by the χ² test. (E) DKK1+ and DKK1− samples were clustered according to the number of metastatic axillary nodes; statistical significance was evaluated by the χ² test. (F) DKK1+ and DKK1− samples were clustered according to the tumour stage grouping (AJCC); statistical significance was evaluated by the χ² test. (G) A crude protein extract was prepared from available tumour samples as described in Materials and Methods. Dickkopf-1 secretion was determined by ELISA. The results presented are the average of at least two independent experiments.
tumours of patients with a higher number of metastatic axillary lymph nodes, specifically with 10 or more invaded nodes (Figure 3E, \( P = 0.002 \)). Additionally, a higher proportion of DKK1 positive tumours was found in advanced breast cancer stages (American Joint Committee on Cancer, TNM stage grouping; Figure 3F). All tumours from patients with stage IIC were DKK1\(^+\) \(( P = 0.04)\), which includes tumours of any size presenting 10 or more metastatic axillary nodes. Finally, although it did not reach statistical significance, DKK1 expression was also documented in some of the most aggressive tumours, namely, in 39% of poorly differentiated histopathological grade 3 tumours and in 31% of tumours wider than 2 cm in their greatest diameter (data not presented). Furthermore, HER-2/new overexpression was observed in only one DKK1-positive tumour. Dickkopf-1 expression was found in lobular as well as in ductal carcinomas and was not associated with recurrence. Altogether, cluster analysis revealed significant preferential DKK1 expression in familial and hormone-resistant breast cancers, which also encompassed the most aggressive tumours.

Importantly, DKK1 protein production was evaluated by ELISA in crude extracts prepared from breast cancer clinical specimens. As shown in Figure 3G, we detected DKK1 protein in four out of the six RT–PCR/DKK1\(^+\) samples. The two samples in which no DKK1 protein was detected had the lowest level of mRNA \((<260\) copies\)). Interestingly, when we evaluated 12 samples that were originally categorised as DKK1\(^+\) by RT–PCR, 11 were negative for the DKK1 protein and one was positive (the mRNA for this sample was prepared from an ER\(\)/PR\(^−\) specimen).

Altogether, these data demonstrate DKK1 production (mRNA and protein) from breast cancer specimens, with a preferential expression pattern in tumours with poor outcomes.

**DKK1 is expressed in multiple tumour types**

We next evaluated if DKK1 was expressed in tumours of other origins. Interestingly, as seen in Figure 4A and B, DKK1 expression was revealed in cell lines derived from lung cancer (five out of five), melanomas (nine out of 11), ovarian cancer (SKOV3) and colon cancer (HCT116). Dickkopf-1 was detected in two prostate cancer lines known to be hormone-independent (DU145 and PC3), but not in LNCaP, which is hormone-dependent (Figure 4B). The latter observation further corroborated previous findings on breast cancers, where DKK1 was preferentially expressed in hormone receptor-negative tumours (Figure 3C). Expression of DKK1 in some cancer cell lines was also confirmed by real-time RT–PCR (Supplementary data, Figure 2). Dickkopf-1 protein secretion was evaluated in culture supernatants. As presented in Figure 4C, secretion was confirmed in cancer cell lines derived from the prostate (PC3), colon (HCT116), lung (H460) and one melanoma (S86mel). Surprisingly, DKK1 was barely detected in two other melanoma lines that were positive for mRNA. We further evaluated DKK1 in cell extracts from these two melanoma lines, but no protein was detected, excluding the possibility that DKK1 is sequestered inside the cell (data not shown).

Finally, as depicted in Figure 4D, expression was evaluated in clinical samples prepared from kidney cancer, and we detected DKK1 at \(>200\) copies/1\(\times10^5\) copies of \(\beta\)-actin in six specimens \((n = 20)\). In addition, DKK1 was found in 11 out of 23 lung cancer specimens (Figure 4E). Altogether, these data suggest that DKK1 is a shared antigen expressed in multiple cancer types.

**DISCUSSION**

Tumours have an aberrant protein expression profile as a consequence of genomic and proteomic alterations. Frequently, genes specialised in embryonic development are abnormally expressed in tumours. We describe here DKK1 expression in human tumours of various origins, including breasts, lungs and kidneys. Dickkopf-1, which is involved in some aspects of embryonic development, was detected in mature human tissues,
mainly in the placenta, an observation reported by Fedi et al. (1999). Interestingly, by analysing its expression profile in breast cancer patients, DKK1 appears in tumours with a poor outcome, specifically hormone-independent cases. Also, we reported preferential tumour expression in women with familial cases of the disease. Finally, we observed substantial DKK1 protein secretion in breast cancer lines, which was further confirmed in crude extracts prepared from breast cancer specimens.

In the embryo, DKK1 functions as a secreted protein interfering with the canonical Wnt pathway (Mao et al., 2002). In the absence of DKK1, Wnt interacts with two co-receptors, namely, LRP5/6 and Fz, which results in ß-catenin accumulation and migration to the nucleus. Consequently, interaction with the transcription factor TCF delivers positive signals for cell proliferation (reviewed in Rothbacher and Lemaire, 2002; Brennan and Brown, 2004). Interestingly, in 1982, Nusse and Varmus (1982) identified the first Wnt gene as being a mammary oncogene, and several members of the Wnt family have been linked to cancer development, especially of the breast (reviewed in Li et al., 2000).

Surprisingly, low levels of membranous ß-catenin expression have been associated with significantly worse outcomes (Dolled-Filhart et al., 2006), which contradicts other studies (Lin et al., 2000; Chung et al., 2004). As such, there is still much debate about the link between tumour aggressiveness and ß-catenin expression. Interestingly, DKK1 negatively affects the Wnt pathway. Interestingly, in 1982, Nusse and Varmus (1982) identified the first Wnt gene as being a mammary oncogene, and several members of the Wnt family have been linked to cancer development, especially of the breast (reviewed in Li et al., 2000).

Consequently, inhibition of the Wnt pathway by DKK1 initiates cardiogenesis early in vertebrate embryos (Marvin et al., 2001; Foley and Mercola, 2005).

Dickkopf-1 has been studied in the context of colon and gastric cancers. In colon cancer, Gonzalez-Sanchez et al. (2005) reported that the loss of DKK1 expression may open the door to cancer by removing the inhibitory effect on the Wnt/ß-catenin pathway. Dickkopf-1 epigenetic inactivation may be a consequence of CpG methylation (Aguilera et al., 2006; Mikata et al., 2006). However, hypermethylation has been observed in only 17% of colon cancer clinical specimens, which indicates that this phenomenon is real but cannot be generalised (Aguilera et al., 2006). Also, the convincing mechanistic demonstration was performed mostly with cancer cell lines treated with the demethylating agent 5-aza-2'-deoxycytidine (DAC) and contradicts the fact that DKK1 is secreted by many highly proliferative cancer cell lines (Figures 1B, 3G and 4C) and is detected in many breast, kidney and lung cancer specimens.

Consequently, DKK1 may have either negative or positive consequences on development, depending on time and tissue distribution during embryogenesis. In cancer, DKK1 expression does not apparently alter cell growth, especially since we noted its distribution during embryogenesis. In cancer, DKK1 expression and currently under investigation. Previous studies have shown that artificial DKK1 expression in some tumour lines with poor prognosis. The link between DKK1 and Wnt in the context of cancer progression is plausible and currently under investigation. Previous studies have shown that artificial DKK1 expression in some tumour lines with constitutive activation of the ß-catenin pathways resulted in some decrease of cell viability but only in the presence of an oxidative stress inducer (Bafico et al., 2004). In cancer cell lines such as MDA231 and HCT116 where ß-catenin is upregulated, the addition of inhibitors of the canonical Wnt pathway (other than DKK1) led to a marked reduction of free ß-catenin (Bafico et al., 2004; Gregoireff and Clevers, 2005). However, according to our findings, these two cell lines already secrete high levels of DKK1 protein, which is known to be an inhibitor of the canonical Wnt pathway. Consequently, none of these in vitro studies correlate with the clinical observation we report here, about the presence of DKK1 protein in growing tumours from breast cancer patients. It is too early to speculate as to whether DKK1 plays a role in cancer similar to its known function in normal cells and in embryogenesis. It may be possible that DKK1 overexpression in in vitro systems may be masked by its other features when expressed at a physiological level. Still, a high cytoplasmic ß-catenin level was found in patients with poor prognosis (Lin et al., 2000). DKK1 has been linked to other attributes specific to cancer cells. For example, Hall et al. (2005) have recently reported that prostate cancer-derived DKK1 is involved in osteoelastic activity in bone metastases. Dickkopf-1 could also be involved in particular phenotypes of hormone responsive tumours. We observed statistically significant preferential DKK1 expression in hormone receptor-negative (ER-) breast tumours (Figure 3A and C). Dickkopf-1 is produced by progesterone in normal endometrial stroma cells (Tulac et al., 2006), but there is insufficient topical DKK1 expression in normal tissue for it to be linked to the expression profile reported here in breast cancer. Interestingly, Faivre and co-workers recently reported that the Wnt pathway can be upregulated by the progesterone receptor in breast cancer (Faivre and Lange, 2007). However, it is too soon to establish a link between DKK1 expression and the absence of hormone receptors. In fact, we observed DKK1 in two hormone-independent prostate cancer lines (DU4S and PC3; Figure 4B) but not in a hormone-dependent tumour (LNCaP). This expression profile is similar to that observed in breast cancer. Finally, we observed coexpression of DKK1 and HER-2/neu in breast cancer cells in only one out of the 21 DKK1+ tumours (data not shown). Consequently, only one of those tumours would be eligible for treatment with Herceptin™, an antibody interfering with tumour progression. This further emphasises the necessity of finding additional targets for immunotherapy.

Interestingly, DKK1 could have potential applications as a secreted tumour marker for cancer diagnosis, staging and monitoring of relapse. Additional investigations are required to establish the feasibility of DKK1 protein detection in different body specimens or fluids.

In conclusion, as DKK1 is specifically expressed in common cancers, and absent from essential normal tissues, this protein is a potential TA for cancer immunotherapy. Its role as an inhibitor of the Wnt canonical pathway in normal cells aside, it may be possible to target DKK1 for a cytotoxic response through CD8 T-cell recognition as a consequence of internal antigen processing leading to MHC class I presentation. In addition, a humoral response may be involved, as antigen-presenting cells can take up secreted tumour-derived DKK1 and elicit a CD4+ helper T-lymphocyte response. Importantly, considering that DKK1 is preferentially expressed in tumours from women with a family history, but absent from important normal tissues, the protein could be targeted in a preventive vaccine for women at risk of developing the condition. Actually, about 70–80% of women at high risk for breast cancer are predicted to develop the disease and, presently, with the exception of radical mastectomy, no effective prevention strategies are available.

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**REFERENCES**

Aguilera O, Fraga MF, Ballestat E, Paz MF, Herranz M, Espada J, Garcia JM, Munoz A, Esteller M, Gonzalez-Sancho JM (2006) Epigenetic inactivation of the Wnt antagonist DICKKOPF-1 (DKK-1) gene in human colorectal cancer. *Oncogene* 25: 4116 – 4121

Bafico A, Liu GZ, Goldin L, Harris V, Aaronson SA (2004) An autocrine mechanism for constitutive Wnt pathway activation in human cancer cells. *Cancer Cell* 6: 497 – 506

Brennan KR, Brown AMC (2004) Wnt proteins in mammary development and cancer. *J Mammm Glind Biol Neoplasia* 9: 119 – 131

Chung GG, Zerkowski MP, Ocal IT, Dolled-Filhart M, Kang JY, Psyrri A, Brennan KR, Brown AMC (2004) Wnt proteins in mammary development and cancer. *J Mammm Glind Biol Neoplasia* 9: 119 – 131

Dolled-Filhart M, McCabe A, Giltnane J, Cregger M, Camp RL, Rimm DL (2004) Quantitative in situ analysis of beta-catenin expression in breast cancer shows decreased expression is associated with poor outcome. *Can Res* 66: 5487 – 5494

Li Y, Hively WP, Varmus HE (2000) Use of MMTV-Wnt-1 transgenic mice for studying the genetic basis of breast cancer. *Oncogene* 19: 1002 – 1009

Lin SY, Xie WY, Wang JC, Kwong KY, Spohn B, Wen Y, Pestell RG, Hung MC (2000) Beta-catenin, a novel prognostic marker for breast cancer: Its roles in cyclin D1 expression and cancer progression. *Proc Natl Acad Sci USA* 97: 4262 – 4266

Mao BY, Wu W, Davidson G, Marhold J, Li MF, Mehler BM, Delius H, Hoppe D, Stannek P, Walter C, Glimika A, Niehre S (2002) Kremen proteins are Dickkopf receptors that regulate Wnt/beta-catenin signaling. *Nature* 417: 664 – 667

Marvin MJ, Di Rocco G, Gardiner A, Bush SM, Lassar AB (2001) Inhibition of Wnt activity induces heart formation from posterior mesoderms. *Genes Dev* 15: 316 – 327

Mikata R, Yokosuka O, Fukai K, Imazeki F, Arai M, Tada M, Kuribara T, Zhang KY, Kanda T, Saitoh H (2006) Analysis of genes upregulated by the demethylating agent 5-aza-2’d-deoxycytidine in gastric cancer cell lines. *Int J Cancer* 119: 1616 – 1622

Mukhopadhyay M, Shtrom S, Rodriguez-Esteban C, Chen L, Tsukui T, Gomer L, Dorward DW, Glimika A, Grinberg A, Huang SP, Niehre S, Belmonte JC, Westphal H (2001) Dickkopf1 is required for embryonic head induction and limb morphogenesis in the mouse. *Dev Cell* 1: 423 – 434

Nusse R, Varmus HE (1982) Many tumours induced by the mouse mammary-tumour virus contain a provirus integrated in the same region of the host genome. *Cell* 31: 99 – 109

Rothbacher U, Lemaire P (2002) Creme de la Kremen of Wnt signalling. *Nat Cell Biol* 4: E172 – E173

Selten MV, Tamai K, Brott BK, Kuhl M, Sokol S, He X (2001) Head inducer Dickkopf-1 is a ligand for Wnt coreceptor LRP6. *Carr Biol* 11: 951 – 961

Strausberg RL, Buetow KH, Emmert-Buck MR, Klausner RD (2000) The cancer genome anatomy project – building an annotated gene index. *Trends Genet* 16: 103 – 106

Takata K (1990) alpha-fetoprotein: reevaluation in hepatology. *Hepatology* 12: 1420 – 1432

Tian E, Zhan FH, Walker R, Rasmussen E, Ma YP, Barlogie B, Shaughnessy JD (2003) The role of the Wnt-signaling antagonist DKK1 in the development of osteolytic lesions in multiple myeloma. *N Engl J Med* 349: 2483 – 2494

Tulac S, Overgaard MT, Hamilton AE, Jumle NL, Suchaneck E, Giudice LC (2006) Dickkopf-1, an inhibitor of Wnt signaling, is regulated by progesterone in human endometrial stromal cells. *J Clin Endocrinol Metab* 91: 1453 – 1461

Wirths O, Waha A, Wegen S, Schirmacher P, Kuhne T, Goodyer CG, Albrecht S, von Schweinitz D, Pietsch T (2003) Overexpression of human Dickkopf-1, an antagonist of wingless/WNT signaling, in human hepatoblastomas and Wilms’ tumours. *Lab Invest* 83: 429 – 434