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Antiproliferative Activity of REIC/Dkk-3 and Its Significant Down-Regulation in Non-Small-Cell Lung Carcinomas

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We recently reported the cloning of the *REIC/Dkk-3* gene, whose expression was shown to be down-regulated in many human immortalized and tumor-derived cell lines [T. Tsuji *et al.* (2000) *Biochem. Biophys. Res. Commun.* 268, 20–24]. In the present study, we demonstrated that expression of the exogenous *REIC/Dkk-3* gene in tumor cells inhibited cell growth. Furthermore, the level of REIC/Dkk-3 mRNA in normal human cells was lowest in the late G_1 phase during the cell cycle. Then we found that the expression of REIC/Dkk-3 was significantly down-regulated in surgically resected non-small-cell lung carcinomas. We determined the *REIC/Dkk-3* locus on chromosome 11p15, where loss of heterozygosity has frequently been observed in human tumors. These findings indicate that REIC/Dkk-3 may function as a tumor suppressor.

We previously reported the cloning of the *REIC/Dkk-3* gene, which is a member of the Dickkopfs (*Dkks*) gene family, and we demonstrated that the expression of REIC/Dkk-3 was markedly decreased in human immortalized and tumor-derived cell lines (1). Dkks are secretory glycoproteins that are composed of at least five family members. Recent studies have shown that some Dkks (Dkk-1 and -4) play a role in induction of amphibian head structures and that their effects seem to be mediated by their ability to antagonize Wnt signaling (2, 3).

There is evidence that several components of the Wnt signaling pathway have been implicated in human tumors or experimental cancer models. Wnt was originally identified as an oncogene activated by the mouse mammary tumor virus in murine breast cancer (4). Furthermore, several members of the Wnt family induced morphological alteration and increased saturation density of mammary epithelial and fibroblast cells (5). Similarly, genetic alterations of APC (adenomatous polyposis coli) and β-catenin, negative and positive regulators in the Wnt signaling pathway, respectively, have been observed in human colon cancer (6), melanomas (7), and hepatocellular carcinomas (8). In addition, mutations in the human *Axin1* gene, a negative regulator of the Wnt pathway, were found in human hepatocellular carcinomas (9). These findings indicate that activation of Wnt signaling pathways may be related to the development of some human cancers.

In the present study, we evaluated growth-inhibitory activity of REIC/Dkk-3 toward human tumor cells. We found that REIC/Dkk-3 has the ability to inhibit the proliferation of human tumor cells. Furthermore, we showed that the expression of REIC/Dkk-3 mRNA is markedly down-regulated in non-small-cell lung carcinomas (NSCLC).

MATERIALS AND METHODS

Cell lines and tissues. Normal human diploid fibroblasts (KMS-6) were cultured in Eagle minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS, Intergen, NY), 2 mM glutamine and 100 μg/ml kanamycin. The cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 and subcultured with 0.2% trypsin plus 0.02% EDTA in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) when they had reached confluence. Tumor and noncancerous tissues were obtained with informed consent, from 34 patients who had undergone surgical operations at the Okayama University Medical School Hospital.

Transfections and measurement of [^{3}H]thymidine uptake. For transfection analysis, REIC/Dkk-3 cDNA was subcloned in a pTracer-EF vector (Invitrogen, San Diego, CA). Saos-2 cells were plated at 5×10^4 per well in 24-well plates. After 12 h, cultures were transfected with 2 μg of plasmid DNA (pTracer/REIC/Dkk-3) by the calcium phosphate method. For measurement of DNA synthesis, cells were labeled with 1 μCi/well of [methyl-^{3}H]thymidine (1 Ci/mmol) for 2 or 12 h and then washed *in situ* twice each with ice-cold PBS, 5% trichloroacetic acid and 95% ethanol. The cells were then

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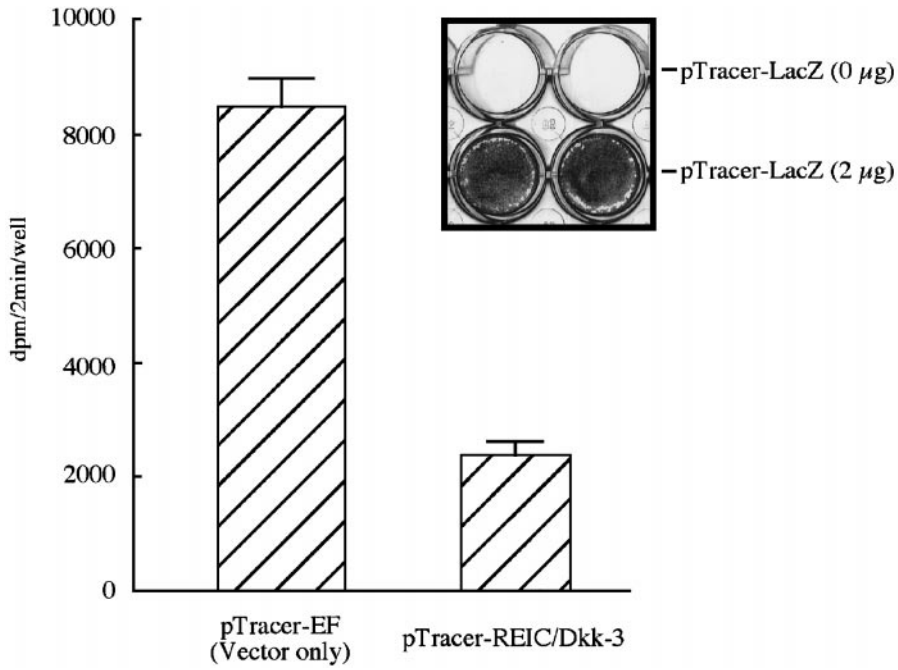


FIG. 1. Growth inhibitory effects of the REIC/Dkk-3 on tumor cells. Human osteosarcoma Saos-2 cells were plated at 5×10^4 per well in 24-well plates. After Saos-2 cells had been transfected with 2 μg of plasmid DNA by the calcium phosphate method, they were labeled for 12 h with [^{3}H]thymidine. Vertical bars indicate SD. Transfective efficiency was measured by X-gal staining of LacZ-transfected Saos-2 cells.

lysed with 200 μl of 0.3 N NaOH. Aliquots of the cell lysates were neutralized with 1 N HCl and the radioactivity was measured in a liquid scintillation counter.

Northern blot analysis. Total cellular RNA was extracted by the guanidinium thiocyanate-phenol method. Total cellular RNA (10 μg) was fractionated by electrophoresis through a formaldehyde-agarose gel and transferred to a Hybond N$^+$ membrane (Amersham Pharmacia Biotech, UK). The membrane was hybridized at 42° with a probe in solution containing 5× SSC, 50% formamide, 1′ Denhardt's solution, 20 mM sodium phosphate (pH 6.8), 5 mM EDTA, 0.2% SDS and 100 μg/ml sheared heat-denatured salmon sperm DNA. The cDNA probe was labeled with [α-^{32}P]dCTP by a random priming reaction. The signal intensities of Northern blots were quantified using the NIH image software.

Chromosomal localization. The REIC/Dkk-3 chromosome localization was determined by PCR analysis utilizing a hamster-human somatic cell hybrid panel. A commercially produced radiation hybrid panel consisting of various hamster-human hybrids was obtained from Research Genetics (AL). The primers used were 5′-GATTTAGATCTGGACCAGGC-3′ (1244–1263 nucleotides [nt]) and 5′-CTGAGCAACACTGCTGGATG-3′ (1777–1796 nt), producing a 553-nt DNA fragment or 5′-TACTGTTAGGAACAGCAGTGT-3′ (2129–2149 nt) and 5′-GGTGGCAACAGTCCATGACACCTG-3′ (2457–2480 nt), yielding a 352-nt DNA fragment. PCR conditions were initial denaturation at 94°C for 3 min followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 63°C for 30 s, and extension at 72°C for 1 min. PCR products were separated on 2% agarose gels and stained with ethidium bromide.

RESULTS

Growth Inhibitory Effects of the REIC/Dkk-3 Gene on Tumor Cells

To investigate whether REIC/Dkk-3 could inhibit growth of human tumor cells, we transfected a human osteosarcoma Saos-2 cell line with pTracer-REIC/Dkk-3. As shown in Fig. 1, the transfection efficiency of Saos-2 cells with pTracer-Lac-Z was more than 50%. Transient expression of exogenous REIC/Dkk-3 significantly decreased DNA synthesis of Saos-2 cells to 28% of that in control cells transfected with the vector alone (Fig. 1). These results indicate that REIC/Dkk-3 has the ability to inhibit the growth of human tumor cells.

Since it has been reported that overexpression of some Wnt-signal-related genes (*Axin1* and *APC*) induces apoptosis in tumor cells (9, 10), we tested whether apoptosis could occur in the *REIC/Dkk-3*-transfected Saos-2 cells by the TUNEL staining method (11). However, we could not detect TUNEL-stained cells in the *REIC/Dkk-3*-transfected Saos-2 cells (data not shown). Thus, we concluded that inhibition of DNA-synthesis in Saos-2 cells was not due to apoptosis.

Relationship between Expression of REIC/Dkk-3 and Cell Cycle Progression

To investigate the relationship between expression of REIC/Dkk-3 and cell cycle progression, we made human normal fibroblasts KMS-6 quiescent by serum starvation and then stimulated the cells by the addition of serum. Total RNA was extracted from the cells harvested at various intervals after serum stimulation and was analyzed by Northern blot. It was found that the expression of REIC/Dkk-3 gradually decreased and

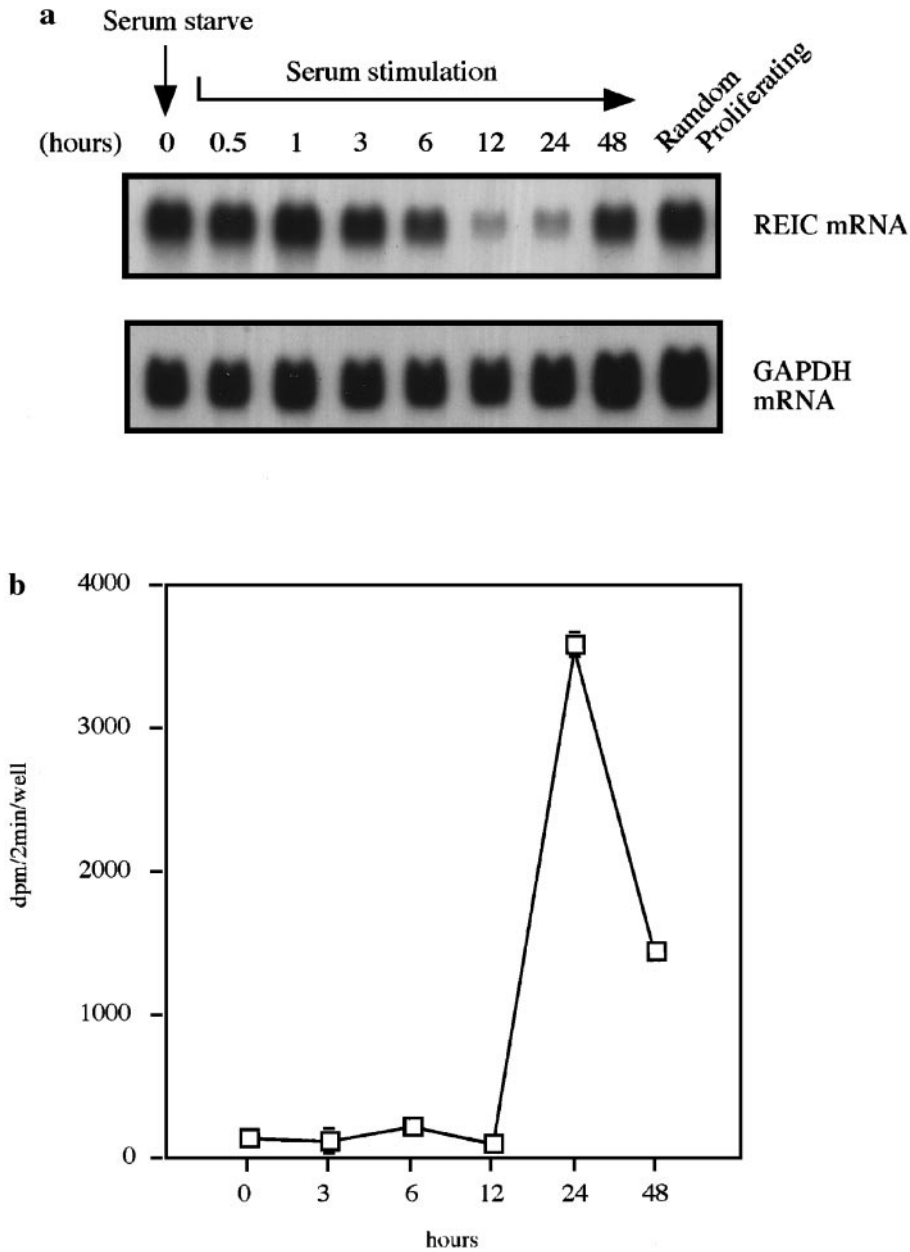


FIG. 2. Normal human fibroblasts down-regulate REIC/Dkk-3 mRNA in the G1/S phase during the cell cycle. (a) Kinetics of REIC/Dkk-3 mRNA during the cell cycle. Normal human KMS-6 fibroblasts were synchronized by serum starvation, stimulated by the addition of serum, harvested at various times after serum stimulation, and analyzed by Northern blot. Upper panel: REIC/Dkk-3 mRNA, Lower panel: GAPDH mRNA for a loading control. (b) [^{3}H]Thymidine uptake by serum stimulation in normal human fibroblasts. The normal human KMS-6 fibroblasts were synchronized by serum starvation, stimulated by the addition of serum, and labeled for the last 2 h with [^{3}H]thymidine after serum stimulation. Vertical bars indicate SD.

that the lowest level was at 12 h after the serum stimulation (Fig. 2a). DNA synthesis in KMS-6 fibroblasts increased at around 24 h after serum addition (Fig. 2b). Thus, the decrease in the expression of REIC/Dkk-3 mRNA preceded the increase in DNA synthesis in KMS-6 cells. These results indicate that the level of REIC/Dkk-3 mRNA in normal human cells was lowest in the late G_1 phase during the cell cycle and that it may be closely related to regulation of the cell cycle.

Expression of REIC/Dkk-3 in Human Cancer Cells

We previously showed that expression levels of REIC/Dkk-3 were remarkably low in many human tumor-derived cell-lines [Hep3B and HuH-7 HCCs, HuH-6 Clone 5 hepatoblastoma, HuCCT-1 cholangiocarcinoma, A549 lung cancer, HaCaT immortalized keratinocytes, HeLa cervical carcinoma, and Saos-2 osteosarcoma] (1). Thus, we investigated whether the

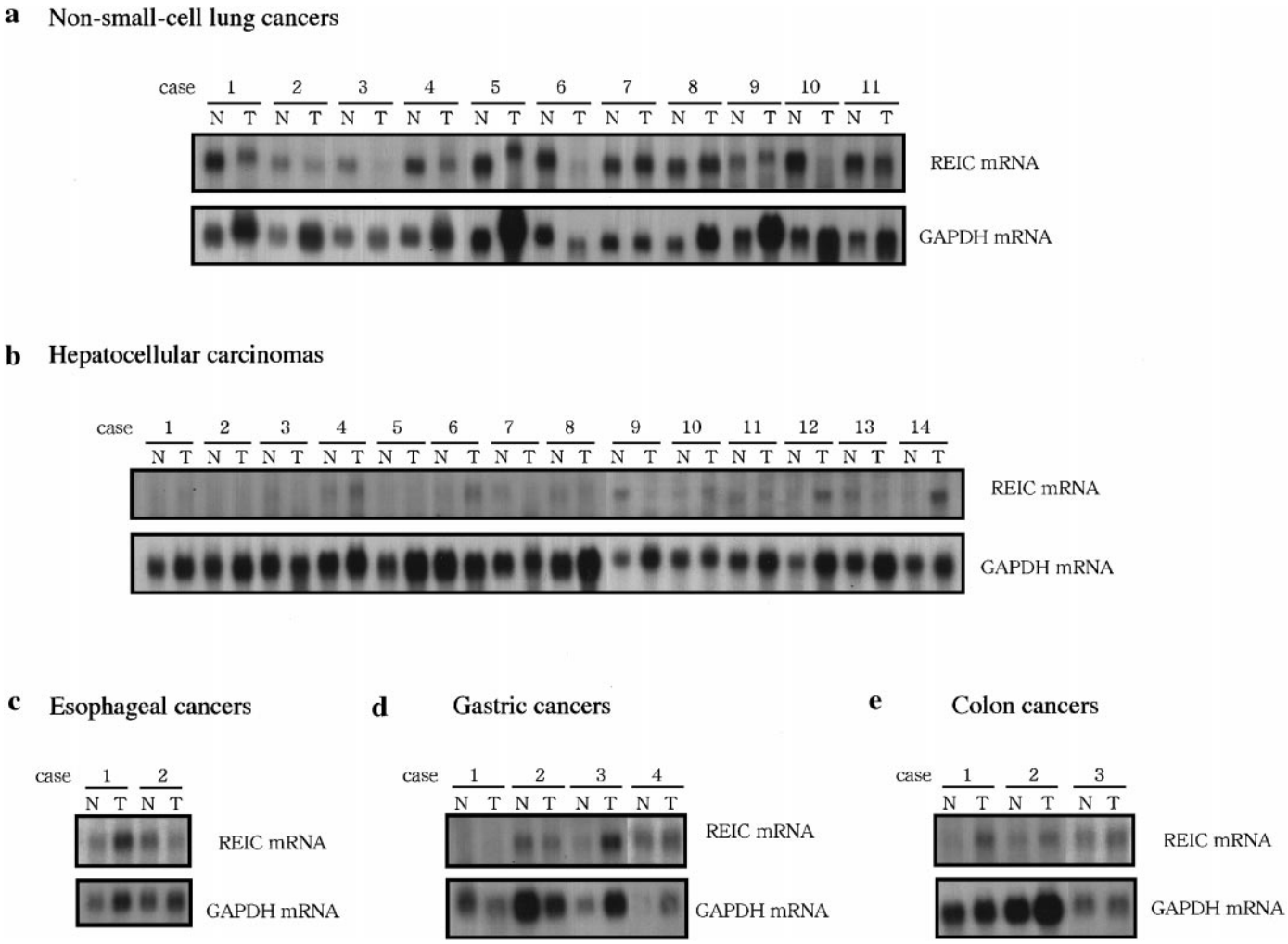
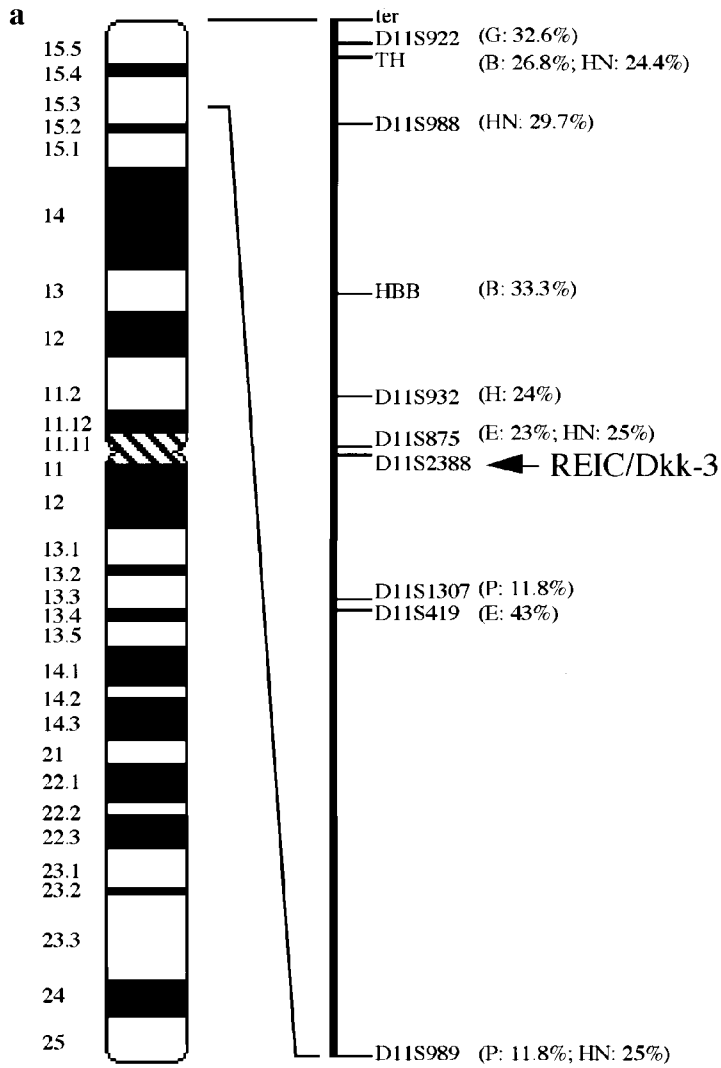


FIG. 3. Reduced expression of REIC/Dkk-3 mRNA in several kinds of surgically resected human tumor tissues compared to their non-cancerous counterparts. a, non-small-cell lung cancers; b, hepatocellular carcinomas; c, esophageal cancers; d, gastric cancers; e, colon cancers. Each upper panel: Total RNA (10 μg) was fractionated by electrophoresis and hybridized with ^{32}P-labeled REIC/Dkk-3 cDNA. Each lower panel: The blot was rehybridized with a probe for GAPDH as a loading control.

expression of REIC/Dkk-3 mRNA is also down-regulated in surgically resected human tumor tissues. We screened five kinds of tumor and their noncancerous counterparts derived from 34 patients with lung, liver, esophageal, stomach, or colon cancer. Northern blot analysis revealed significantly reduced expression of REIC/Dkk-3 mRNA in 10 out of 11 non-small-cell lung carcinomas (NSCLC) examined, compared with their noncancerous counterparts (case 1: 75.0%, case 2: 97.8%, case 3: 99.7%, case 4: 74.2%, case 5: 64.1%, case 6: 87.5%, case 8: 42.1%; case 9: 59.9%; case 10: 78.7%; case 11: 46.5% reduction) (Fig. 3a). We also found down-regulated expression of REIC/Dkk-3 mRNA in 4 out of 13 HCCs (Fig. 3b, case 8: 42.6%, case 9: 99.94%, case 11: 47.7%, case 13: 86.9% reduction), 1 out of 2 esophageal cancers (Fig. 3c, case 2: 77.6% reduction) and 1 out of 4 gastric cancers (Fig. 3d, case 4: 92.5% reduction). Among these tumors, some showed overexpression of REIC/Dkk-3 mRNA (Fig. 3b, cases 6, 10, and 14; Fig. 3e, case 1). On the other hand, there was no significant difference between the expression levels of REIC/Dkk-3 mRNA in colon cancers and in their noncancerous counterparts (Fig. 3e).

LOH of the REIC/Dkk-3 Gene in Human Tumor Cell Lines

We next determined the chromosomal localization of the *REIC/Dkk-3* gene by PCR analysis of a somatic cell hybrid mapping panel using primers for the 3′-noncoding region of REIC/Dkk-3 cDNA. As a result, the *REIC/Dkk-3* gene was detected in the hybrid containing human chromosome 11p15 (Fig. 4a). The same results were also obtained with a second set of the REIC/Dkk-3 gene-specific primers. Moreover, searches of the GenBank for STS genetic markers revealed that



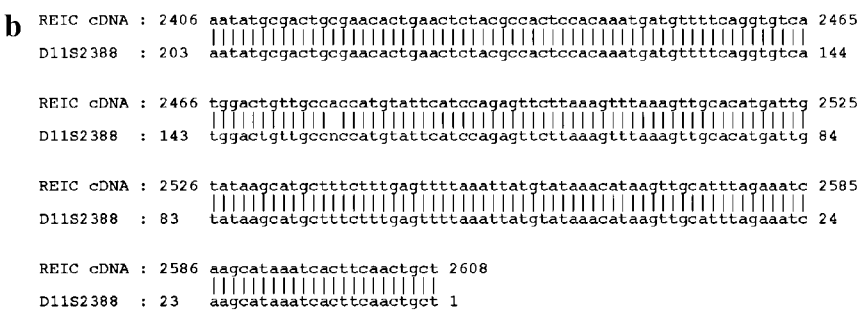


FIG. 4. Relationships of localization between *REIC/Dkk-3* locus and other genetic STS markers on human chromosome 11p15. (a) Frequencies of LOH at 10 *loci* of 11p15 in several kinds of human tumor tissues are shown in parentheses [E, esophageal cancers (12); HN, head and neck cancers (13); G, gastric cancers (16); B, breast cancers (17); P, prostate cancers (18)]. The order of STS markers on chromosome 11 was obtained from a genomic database in Southampton University (http://cedar.genetics.soton.ac.uk/pub/chrom11/map.html). (b) Alignment of the *REIC/Dkk-3* gene from the 3′-noncoding region of REIC/Dkk-3 cDNA (nt 2406-2608 in Accession No. AB034203) and the genetic marker *D11S2388* on human chromosome 11.

the 3′-noncoding region of REIC/Dkk-3 cDNA (nt 2406–2608 in Accession No. AB034203) corresponded to the genetic marker *D11S2388*, which is located on human chromosome 11p15 (Fig. 4b).

DISCUSSION

We recently cloned the *REIC/Dkk-3* gene, whose expression was shown to be markedly down-regulated in many human immortalized and tumor-derived cell lines (1). In the present study, we demonstrated that the expression of the REIC/Dkk-3 was also down-regulated in several kinds of surgically resected human tumor tissues, especially in NSCLC. By transfection experiments, we determined that REIC/Dkk-3 possesses an antiproliferative activity against tumor cells. Furthermore, we found that the *REIC/Dkk-3* gene is localized on chromosome 11p15. In addition, we found that the 3′-noncoding region of REIC/Dkk-3 cDNA corresponds to the genetic marker *D11S2388*.

LOH of several genetic markers frequently occurs near the *REIC/Dkk-3* locus. As shown Fig. 4a, the locus, *D11S875* quite near *REIC/Dkk-3*, shows a high frequency of LOH in esophageal (23%) and head-and-neck carcinomas (25%) (12, 13). Furthermore, LOH on chromosome 11p15 frequently occurs in several types of solid neoplasms, including non-small-cell lung carcinoma (14), hepatocellular carcinoma (15), gastric tumor (16), esophageal tumor (12), head and neck carcinoma (13), breast tumor (17), prostate tumor (18) and ovarian tumor (19). Moreover, functional studies using chromosome-mediated gene transfer revealed that human chromosome 11p15 suppresses the growth of rhabdoid and lung cancer cells (20, 21) and the tumorigenicity of BKV-transformed cells (22). Thus, human chromosome 11p15 is thought to contain an unknown tumor suppressor gene. In addition to LOH, silence of the gene expression by genomic imprinting on chromosome 11p15 has frequently been reported in many kinds of cancer (23). Concerning the gene that is mapped on chromosome 11p15 and encodes a cyclin-dependent inhibitor $p57^{KIP2}$, its paternal allele is quite often imprinted in human brain and some embryonal tumors (24). Thus, reduced expression of REIC/Dkk-3 in tumor cells may be due to LOH or direct genomic imprinting in this region. Taken together, REIC/Dkk-3 may be one of the tumor suppressors.

Regarding the Dkk gene family, it has been reported that *Xenopus* Dkk-1 inhibits Wnt-8 activities in *Xenopus* embryos and may play a role in induction of amphibian head structures (2). Co-microinjection of Dkk-4 mRNA with Wnt-8 mRNA into *Xenopus* eggs also inhibited Wnt-induced axis duplication (3). Similarly, co-expression of human Dkk-1 with Wnt-2 in NIH3T3 cells caused reversion of Wnt-2-induced morphological alterations and drastically inhibited the Wnt-induced increase in β-catenin (25). Thus, some of the Dkk family members are likely to inhibit the β-catenin-dependent Wnt signaling pathway. However, REIC/Dkk-3 had no effect on the axis-inducing activity of Wnt-8, Wnt-2b and Wnt-3a (3). Neither could we observe inhibition of nuclear transport cytoplastic accumulation of β-catenin in REIC/Dkk-3-transfected cells. This indicates that REIC/Dkk-3 may not be involved in the β-catenin-dependent Wnts signaling pathway (data not shown). Thus, REIC/Dkk-3 may have characteristics distinct from other Dkk family members.

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