Biochemical and Biophysical Research Communications **276,** 264–270 (2000)
doi:10.1006/bbrc.2000.3449, available online at http://www.idealibrary.com on IDEAL®

Amplification and Overexpression of TGIF2, a Novel Homeobox Gene of the TALE Superclass, in Ovarian Cancer Cell Lines[1]

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Received August 11, 2000

Homeodomain transcription factors play important roles in directing cellular proliferation and differentiation. A TALE-superclass homeodomain protein, multifunctional repressor of TGFβ-induced transcription. Here we report identification of TGIF2, a novel TALE-superclass homeodomain protein that shows distinct homology with TGIF, especially in its DNA-binding domain. TGIF2 is expressed ubiquitously in human tissues, with the highest levels being found in heart, kidney, and testis. The TGIF2 product contains a putative nuclear localization signal; translocation of the protein to the nucleus was confirmed by transfection of epitope-tagged cDNA. TGIF2 lies on chromosome 20q11.2-12. Since amplification of 20q is often observed among ovarian cancers, we determined the status of DNA copy-number and expression of TGIF2 in 14 ovarian-cancer cell lines. This gene was overexpressed in all lines that showed amplification by FISH analysis. The results suggested that TGIF2 may play an important role in the development and/or progression of some ovarian tumors through a mechanism of gene amplification.

Key Words: **TGIF2; amplification; overexpression; 20q11.2-12; ovarian cancer.**

Homeodomain transcription factors play fundamental roles in directing cellular proliferation and differentiation, and in determining cell fates. Homeodomain proteins can be grouped into multiple subfamilies, on the basis of criteria such as the primary sequences of the homeodomains, flanking sequences, and organization into gene clusters (1). One sub-family of homeobox genes encodes proteins with atypical homeodomains referred to as TALE (three-amino-acid loop extension) (2, 3). Insertion of three amino acids between helices one and two of the homeodomain does not appear to affect DNA binding, but it may play a role in determining specific interactions with other transcription factors (4). Members of this growing sub-family have been identified in diverse species (3) including the human, e.g., Pbx1, Meis1, and TGIF proteins (2, 5–7). Cooperative function among some members of the TALE family is critical for regulating transcription (8–10), and several of them are essential contributors to the Hox-mediated developmental program (11, 12). These features led us to search for novel members the TALE family, in an effort to better understand regulatory mechanisms involving homeodomain proteins.

One of the most interesting members of TALE family is TGIF (5′TG3′ interacting factor). Human TGIF was originally cloned through its ability to bind to a specific retinoid X receptor (RXR)-responsive element (2). TGIF subsequently was identified as a co-repressor that appears to mediate the ability of Smad proteins to negatively regulate transcription (13); expression of TGIF attenuates activation of the TGFβ-responsive reporter and represses Smad-activated transcription. After activation of TGFβ receptor, TGIF interacts with Smad2 and Smad3. This process recruits TGIF to TGFβ-responsive elements and represses TGFβ-activated transcription (13, 14). Repression of Smad-dependent transcription by TGIF correlates with the recruitment of histone deacetylases (HDACs) instead of the co-activator p300 into the Smad complex (13, 14).

[1] This work was supported by grants-in-aid for Cancer Research from the Ministry of Education, Science, Sports, and Culture; from the Ministry of Health and Welfare; from the Agency of Science and Technology; and from the Organization for Pharmaceutical Safety and Research (OPSR), Japan.

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0006-291X/00 $35.00

In the course of searching for novel members of the TALE family by computer-aided screening, we identified a gene whose product we have called TGIF2 because, among the known proteins of the TALE family, it displays the highest degree of homology with TGIF. In view of this distinct homology, TGIF2 protein may, like TGIF, play a role in repressing transcription induced by TGFβ. We mapped the novel gene to chromosome 20q11.2-12. The long arm of chromosome 20 is one of the regions that are amplified most often in solid tumors of humans (15). Recently we (Watanabe *et al.*, submitted) and other groups (16–18) documented frequent amplification of 20q in primary ovarian cancers and cell lines by comparative genomic hybridization. Since one of the functions of TGIF might be corepression of TGFβ-mediated transcription, we investigated whether this gene was a target of amplifications at 20q in the materials we examined, and whether it was over-expressed in ovarian-cancer cell lines.

MATERIALS AND METHODS

Cloning and sequencing of human TGIF2. We searched for TGIF-related proteins by comparing human TGIF sequence (GenBank Accession No. NM_003244) against the databases of ESTs and genomic sequences, using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/). This search identified four EST clones that we then purchased from Incyte Genomics Inc. (St. Louis, MO): IMAGE clones 417146, 1700966, 2469542, and 2964507. Since all these EST clones contain part of entire coding sequence, we used reverse transcription-polymerase chain reaction (RT-PCR) to obtain cDNA fragments containing entire coding sequence. cDNA was synthesized using RNA derived from ovarian-cancer cell line Kuramochi, and then PCR amplifications were performed using primers generated on the basis of EST and genomic sequence (GenBank Accession No. AL05318), and Platinum *Pfx* DNA polymerase (Gibco BRL, Gaithersburg, MD) according to the manufacturer's directions. PCR products were sub-cloned for sequencing with a 377 ABI autosequencer (PE Biosystems, Foster City, CA). Analyses of sequences and comparisons of data were performed using BLAST, Motif (http://motif.genome.ad.jp/) and PSORT II (http://cookie.imcb.osaka-u.ac.jp/nakai/psort.html) programs.

Cell lines. Fourteen ovarian-cancer cell lines, all listed elsewhere (Watanabe *et al.*, submitted), were maintained in RPMI1640 medium supplemented with 10% FCS and penicillin-streptomycin.

Northern blot analysis. Northern blotting was performed as described elsewhere (19). Briefly, 10 μg of total RNA extracted from each ovarian cancer cell line was separated in 1% agarose/0.67 M formaldehyde gel, and then transferred onto a positively charged nylon membrane (Hybond N+, Amersham Pharmacia Biotech, Tokyo, Japan). Northern blots of RNA from different human tissues (Human 12-lane MTN blot and Human MTN blot II) were obtained from Clontech, Inc. (Palo Alto, CA). A cDNA probe containing full coding sequence of *TGIF2* was labeled with [α^{32}P]dCTP by random priming (Megaprime, Amersham Pharmacia Biotech), and hybridized to the prehybridized blots. All blots were washed in a solution of 0.1XSSC/0.1% SDS, and then exposed for 48–84 h.

Expression construct and transfection. A plasmid construct encoding an epitope-tagged form of TGIF2 was assembled by cloning the coding sequence of this gene in-frame with Xpress epitope into the pcDNA3.1/HisC vector (Invitrogen, Carlsbad, CA). After confirming the sequence, we transfected pcDNA3.1-His-TGIF2 into COS-7 cells using Lipofectin (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions. Forty-eight hours after transfection, cells were washed with phosphate-buffered saline and fixed with acetone/methanol (1:1 v/v). Epitope-tagged TGIF2 protein was localized within cells using monoclonal anti-Xpress antibody (Invitrogen) and FITC-conjugated anti-mouse secondary antibody (MBL, Nagoya, Japan) according to the manufacturer's suggestions.

Fluorescence in situ hybridization (FISH). FISH analyses were performed as described previously (20), using a P1-derived artificial chromosome (PAC) containing *TGIF2* (RP5-977B1, GenBank Accession No. AL050318) with or without a bacterial artificial chromosome (BAC) located at 20p11.2 (RP11-11M17) as the probes. Metaphase chromosomes were prepared from normal male lymphocytes and from each ovarian-cancer cell line. Hybridization to normal lymphocyte nuclei was performed to determine the chromosomal location and to ascertain that the probes recognized a single-copy target. FISH signals specific to the probe were examined in 10–15 metaphase spreads from each cell line. The copy-number of *TGIF2* in each cell line was determined on the basis of signals from the *TGIF2*-PAC probe relative to those of the 20p11.2-BAC probe.

RESULTS AND DISCUSSION

TGIF2 Is a Novel Member of Tale Superclass Homeobox Genes

TGIF (5′TG3′-interacting factor) encodes a DNA-binding homeodomain protein that can repress TGFβ-induced and retinoid X receptor-dependent transcription (2, 13, 14). Evidence for the existence of a novel TGIF-related gene was gathered during the process of comparing TGIF amino-acid and nucleotide sequences against public databases using the BLAST program. With this approach, we identified genomic sequence present in PAC RP5-977B1 (GenBank Accession No. AL05318), which contains a gene predicted to encode a protein similar to TGIF, and four expressed sequence tags (ESTs; IMAGE clones 417146, 1700966, 2469542, and 2964507). We used RT-PCR to connect gaps between the partial TGIF-related sequences of the ESTs and to confirm the exonic sequences predicted from the PAC. In this manner we isolated a cDNA that provided the entire coding sequence for a novel 237-amino-acid (29.74 kDa) protein containing an atypical homeodomain referred to as TALE (three-amino-acid loop extension).

The homeodomain of this protein is 77% identical to the corresponding domain within TGIF, but outside this domain the similarity is only 49% (Fig. 1A). Among other functional domains of TGIF (13, 14), only one repression domain (RD-2b) shows moderate homology to the corresponding region within TGIF2. A proline-rich region, thought to be implicated in transcriptional regulation (21), is conserved in the RD-2b domain between the two proteins. This region is also a putative SH3-domain binding site (2). Therefore, TGIF2 may also serve as a transcriptional repressor, although its regulatory activity has not yet been assessed. On the other hand RD-2a, another repression domain of TGIF (14), shows no homology to the corresponding region in TGIF2. Since Wotton *et al.* (13, 14) have reported that this domain is part of a region that interacts with Smads, it will be of great interest to evaluate whether

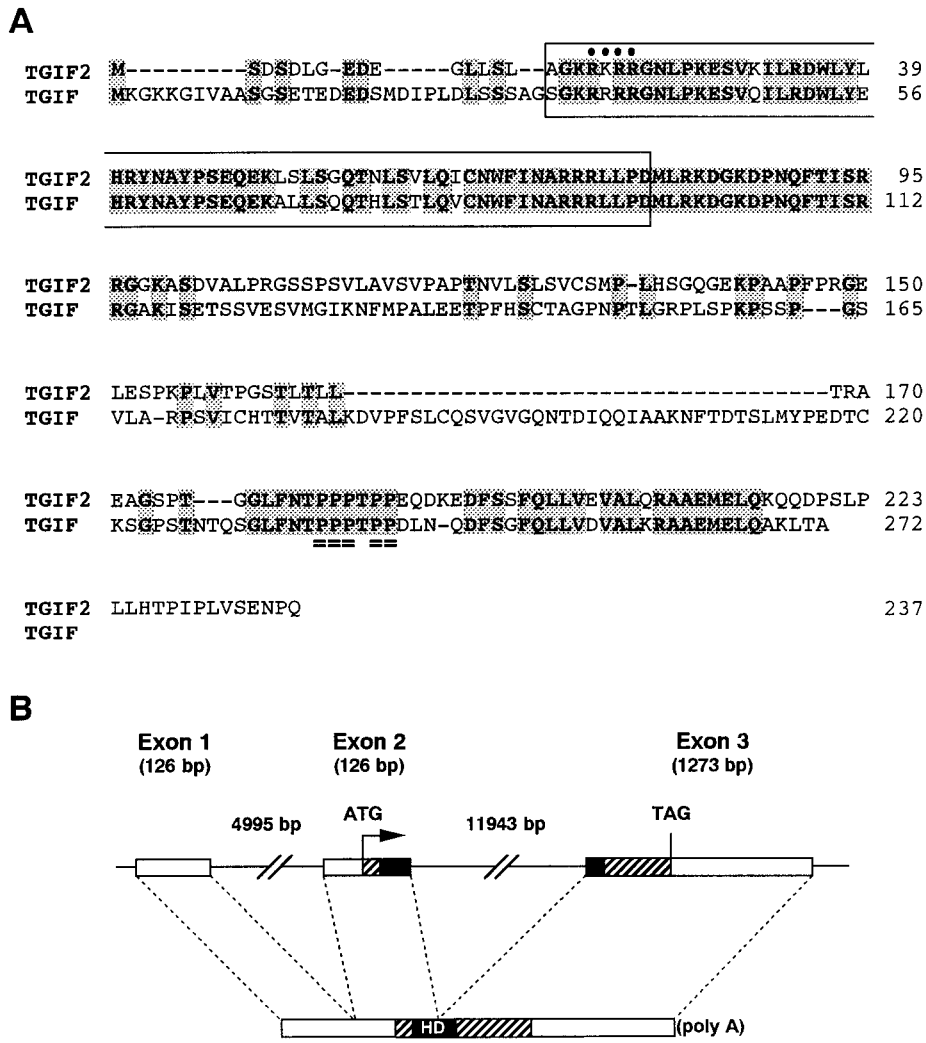


FIG. 1. (A) Alignment of human TGIF and TGIF2 protein sequences (GenBank Accession Nos. NM_003244 and AB042646, respectively). Numbers at right refer to amino-acid residues. Identical amino acids are highlighted by shading. The atypical homeodomain sequences are boxed; double-underlined prolines indicate a putative SH3-domain binding site. A putative nuclear localization signal is indicated by a series of solid circles. (B) Genomic structure of the *TGIF2* gene. ATG (arrow) and TAG, start and stop sites. Open, hatched, and black boxes indicate untranslated, coding, and homeodomain sequences respectively; lines represent introns. The numbers of base pairs in each exon and intron, calculated from cDNA and genomic sequences, are indicated.

TGIF2 can also bind with Smad proteins and, if not, to identify other proteins that do interact with TGIF2.

By comparing our *TGIF2* cDNA sequence with genomic sequence in the PAC, we determined exon-intron boundaries. An interesting feature of the genomic structure of *TGIF2* (Fig. 1B) is the presence of an intron that interrupts the homeodomain coding sequence. This type of intron, first identified in the Ant-C *labial* (*lab*) gene of *D. melanogaster*, is known as a *lab*-class intron (22). It defines an extended family, and homeobox genes in this family appear to be quite ancient based on their presence in widely diverse species.

Expression Pattern of TGIF2

We performed Northern blot analysis of RNA derived from various human tissues to gain insight into the spatial distribution of TGIF2 mRNA. As shown in Fig. 2, a single TGIF2 transcript of ubiquitously expressed in human tissues. The highest levels were found in heart, kidney, and testis, and the transcript was almost undetectable in brain or prostate. Note that this expression pattern is slightly different from that of TGIF; for example, the latter is prominently expressed in placenta, liver, kidney, prostate, ovary, and testis but only weakly in heart, brain, skeletal muscle, or peripheral leukocytes (2).

Nuclear Localization of TGIF2

Since a computer-based analysis using the PSORT II program predicted nuclear localization, we investigated the sub-cellular location of this protein by adding an epitope-tag that would be detected by immunofluo-

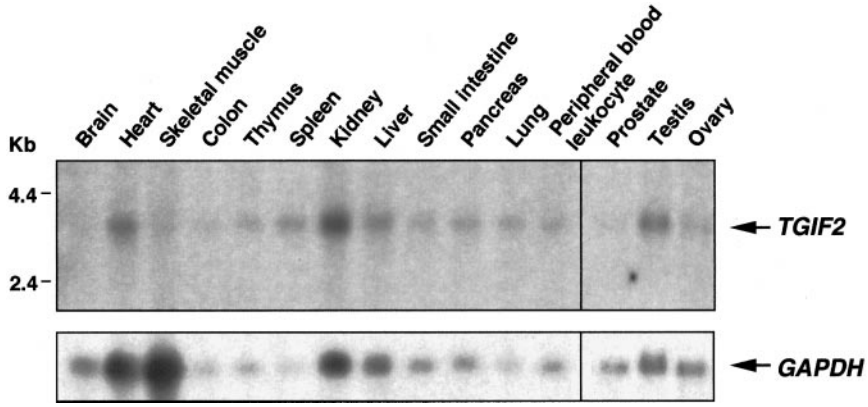


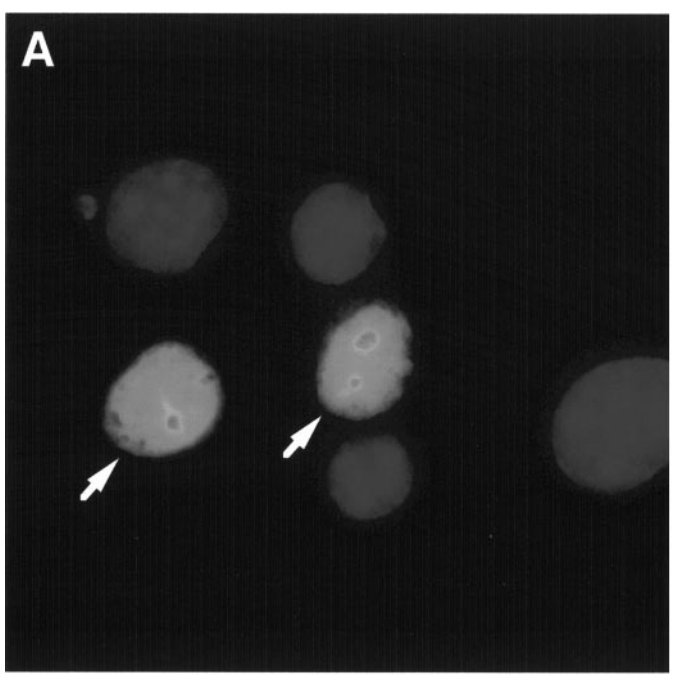
FIG. 2. Expression of *TGIF2* mRNA in normal human tissues. A multi-tissue Northern blot (Clontech) was probed with *TGIF2* cDNA. The same blot was rehybridized with a *GAPDH* probe, as a control for RNA loading and transfer.

rescence. Using this approach we confirmed that TGIF2 is present in the cell nucleus but is excluded from nucleoli (Fig. 3A). No detectable staining was observed in cells transfected with the parental plasmid as a control (data not shown). As it contains a DNA-binding motif, TGIF2 may function as a nuclear transcription factor; it will be interesting to determine whether the nuclear localization of TGIF2 is regulated by any stimulatory signals.

Amplification and Overexpression of TGIF2 in Ovarian-Cancer Cell Lines

FISH analysis, using PAC clone RP5-977B1 as a *TGIF2*-specific probe, produced clear signals on chromosomal band 20q11.2-12 (Fig. 3B).

Our previous CGH analyses had demonstrated that cell lines derived from ovarian cancers often show frequent amplification in the 20q region (Watanabe *et al.*,



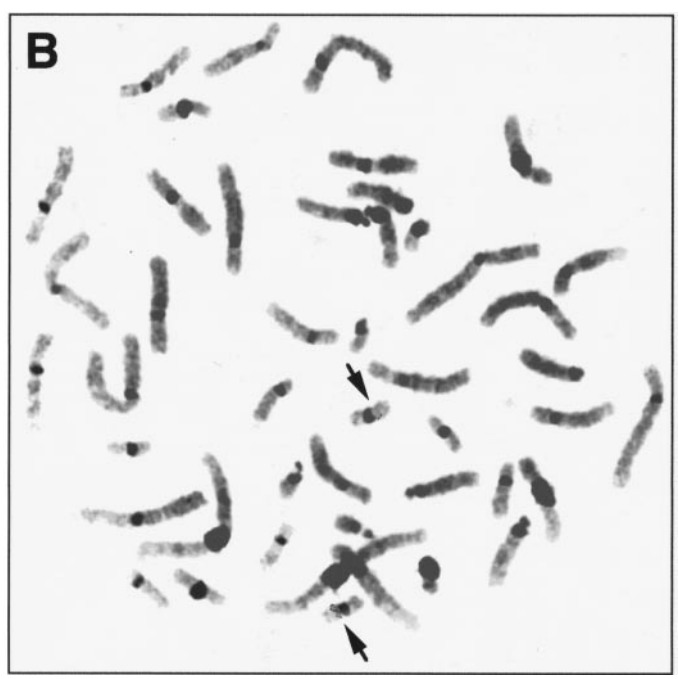


FIG. 3. (A) Nuclear localization of TGIF2. COS-7 cells were transiently transfected with a vector containing Xpress epitope-tagged *TGIF2* cDNA. The presence of tagged protein was detected microscopically after being stained with an anti-Xpress antibody and an FITC-conjugated anti-mouse secondary antibody. The preparations were counter-stained with 4,6-diamidino-2-phenylindole-dihydrochloride (DAPI). Arrows indicate nuclear staining of the TGIF2 construct. (B) Mapping of the *TGIF2* gene by FISH. Metaphase chromosomes from human diploid cells were hybridized with labeled genomic DNA from PAC RP5-977B1. Typical hybridization signals appear on both chromosomes 20 at band 11.2-12 (arrows).

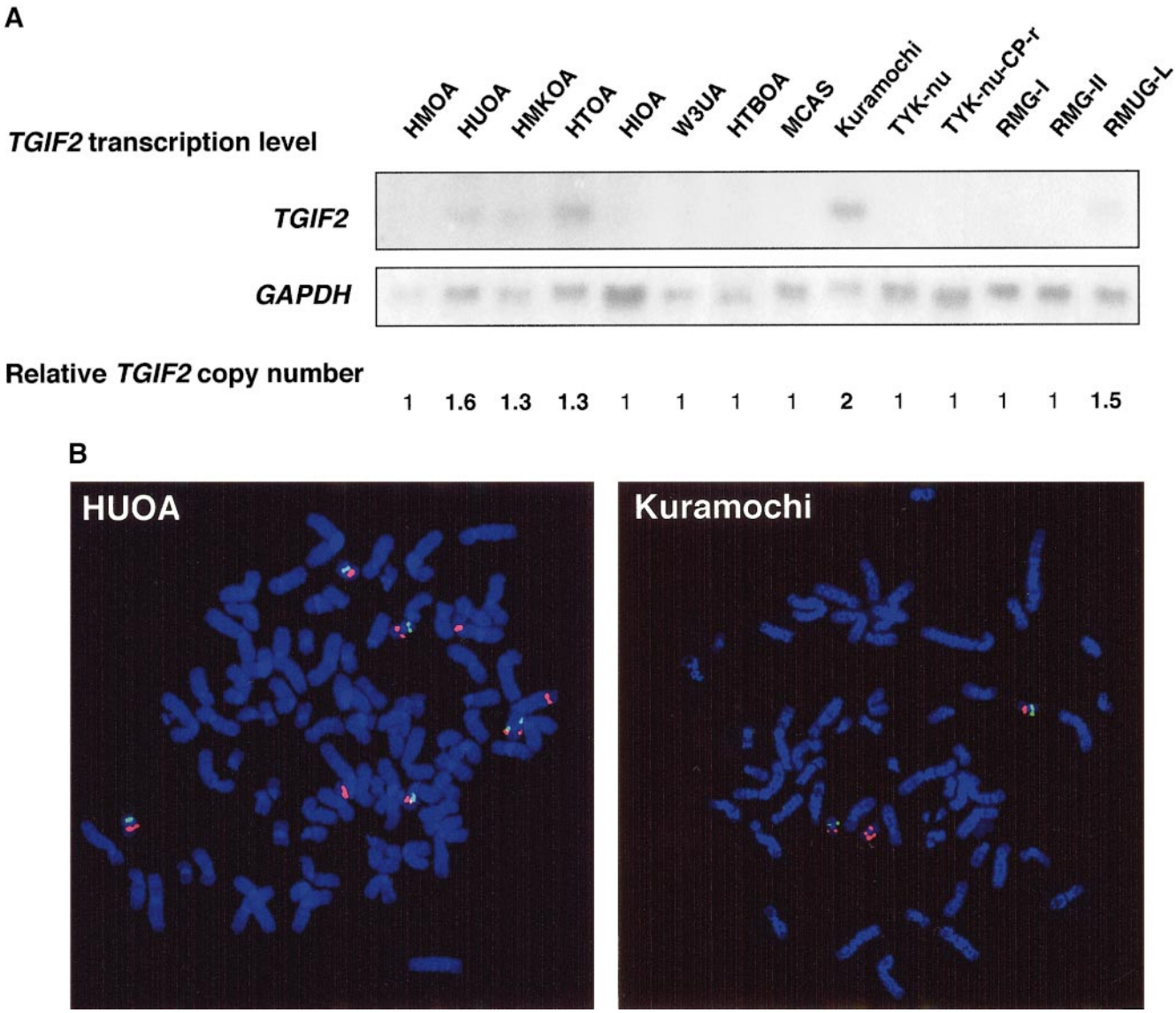


FIG. 4. Over-expression and amplification of the *TGIF2* gene in 14 ovarian cancer cell lines. (A) Northern blot using total RNA from each line, probed with *TGIF2* or *GAPDH* cDNAs. The copy numbers of *TGIF2* relative to a locus on 20p11.2, as detected by FISH, are shown below the relevant lanes. Note that all five cell lines that over-expressed *TGIF2* mRNA also showed increased *TGIF2* copy-numbers. (B) Representative FISH images with the *TGIF2* probe (red) and the 20p11.2 probe (green) hybridized to cells from ovarian cancer lines HUOA and Kuramochi. The preparations were counter-stained with DAPI.

submitted). Although primary ovarian cancers more commonly show amplifications at 20q13 (16–18), frequent gains in DNA copy-number are also observed in a more proximal region that includes 20q11.2-12, especially in advanced tumors. In some breast cancers 20q11.2-12 also shows amplification that is independent of, but highly concomitant with, 20q13 (23, 24). Taken together, these observations suggest that 20q is likely to harbor multiple genes that are activated by an amplification mechanism. These unknown candidate genes may play critical roles in development and/or progression of ovarian cancers, either independently or synergistically. Therefore, we screened 14 ovarian-cancer cell lines for amplification and expression status of the TGIF2 gene, and found over-expression of TGIF2 in five of them (Fig. 4A). The same five cell lines showed amplification of this gene in FISH analysis; mean copy-numbers ranged from 4 to 8 with the increased *TGIF2* copy number relative to a locus at 20p11.2. Two such cell lines are illustrated in Fig. 4B. All cell lines having low levels of TGIF2 expression showed no increases in relative copy-numbers in that region. As these data indicated that TGIF2 can be over-expressed through amplification of DNA at 20q11.2-12, this gene is a strong candidate as one of the targets in the 20q amplicon.

TGIF, the protein with the highest degree of homology to TGIF2, is a transcriptional co-repressor that interacts with Smads to negatively regulate the TGFβ/Smad response in a cell. Since TGFβ is a well-known regulator of cell growth, most frequently acting as a potent growth inhibitor, TGIF may have oncogenic potential through inhibition of TGFβ. Although we have not yet characterized TGIF2 in terms of function, it is possible that this protein acts in a manner similar to TGIF. Acquired resistance to TGFβ-mediated growth inhibition has been proposed as a factor contributing to carcinogenesis in various tissues, including ovary (25); however, alterations of molecules in the TGFβ-signaling pathway, such as Smad4 and TGFβ receptor type II, are rare in ovarian cancers. Further examination will be necessary to determine whether overexpression of TGIF2 is associated with carcinogenesis of ovarian tissues through interference with the normal TGFβ-signaling pathway.

In summary, we have identified *TGIF2* as a novel TALE homeodomain-encoding gene, located at 20q11.2-12. The structure and sub-cellular localization of TGIF2 indicate that this protein functions as a nuclear transcription factor. We are now investigating TGIF2 as a target gene within the 20q amplicon that is frequently seen in ovarian cancers.

ACKNOWLEDGMENTS

We thank Drs. Isamu Ishiwata, Kazunari Kiguchi, Nobuyuki Susumu, Yoshinori Kosugi, and Masaomi Takayama for providing ovarian-cancer cell lines and Professor Yusuke Nakamura for his continuous encouragement.

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