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BRIEF COMMUNICATION

Cloning and characterization of TNKL, a member of tankyrase gene family

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By serological screening of a breast tumor cDNA library we have identified a novel human gene, tnkl, encoding an ankyrin-related protein with a high degree of similarity to tankyrase, the poly(ADP-ribose)polymerase associated with human telomeres (Smith et al, Science 282: 1484). The tnkl gene maps to chromosome 10, while the tnks gene encoding tankyrase is located on chromosome 8. The predicted 1166-aa protein product of the tnkl gene is 78% identical to human tankyrase and 62% to a putative D. melanogaster protein. Since the proteins have essentially identical domain structures, the corresponding genes form a distinct gene family. The possible link between TNKL and cancer justifies its further functional analysis. Genes and Immunity (2001) **2**, 52–55.

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A recently developed cloning strategy SEREX (serological identification of antigens by recombinant expression cloning) is generally applied to identification of potential cancer antigens using patient sera as screening reagents, based on the observation that many tumors elicit humoral immune responses.[1] While the SEREX technique allows to clone antigens to which responses are commonly found in patients with autoimmune disorders, it has proven extremely useful in defining antigens specific for various types of cancer. Additionally, it is becoming apparent that the immune repertoire of cancer patients often identifies gene products that are directly implicated in the pathogenesis of the disease, and thus provides both clues to the mechanism of transformation and potential targets for treatment. Finally, SEREX can be viewed as a general gene discovery tool, since the genes identified in such screens are often expressed at very low levels.

We used this serological cloning strategy to search for novel antigens in breast cancer. A tumor specimen of breast carcinoma was used to isolate and purify poly A^+ RNA. cDNA was synthesized, size selected, and ligated into the Lambda ZAP Express™ vector (Stratagene, La Jolla, CA, USA). Out of 1.8×10^6 plaque forming unit (pfu) in the non-amplified cDNA library, 3×10^5 clones were screened with autologous serum using procedure that allows an easy detection of recombinant tumor-derived polypeptide antigens reactive with high-titer patient IgG.[1,2]

Among positive recombinants we identified and purified a clone MO-BC-203. Extensive serological analysis indicated that the immune response to corresponding gene product occurs in cancer patients and almost never in healthy individuals (manuscript in preparation). The entire 5-kb insert of MO-BC-203 was *in vivo* excised and sequenced (sequence reported in this paper has been deposited to GenBank database, accession number AF264912). A BLAST search indicated very high similarity (83% amino acid identity, see Figure 1) of MO-BC-203 to a putative *D. melanogaster* protein (GenPept accession number AAD34784) and human tankyrase, a poly(ADP-ribose) polymerase (PARP) containing multiple ankyrin repeats and associated with human telomeres (GenPept accession number AAC79841).

The predicted amino acid sequence of MO-BC-203 contains three characteristic hallmarks of tankyrase: the PARP-like catalytic domain, the region homologous to sterile alpha motif, and the ankyrin-related domain (Figures 1 and 2). The PARP domain of MO-BC-203 contains 130 amino acid residues out of 139 aa identical to tankyrase, among which the critical amino acid residues conserved in human and *D. melanogaster* PARPs as well as in prokaryotic ADP-ribosyltransferases, exotoxin A from *P. aeruginosa* and diphteria toxin. The enzymatic activity of a eukaryotically expressed GFP-MO-BC-203 fusion protein was also confirmed by direct autocatalytic radiolabeling of the immunoprecipitated protein product

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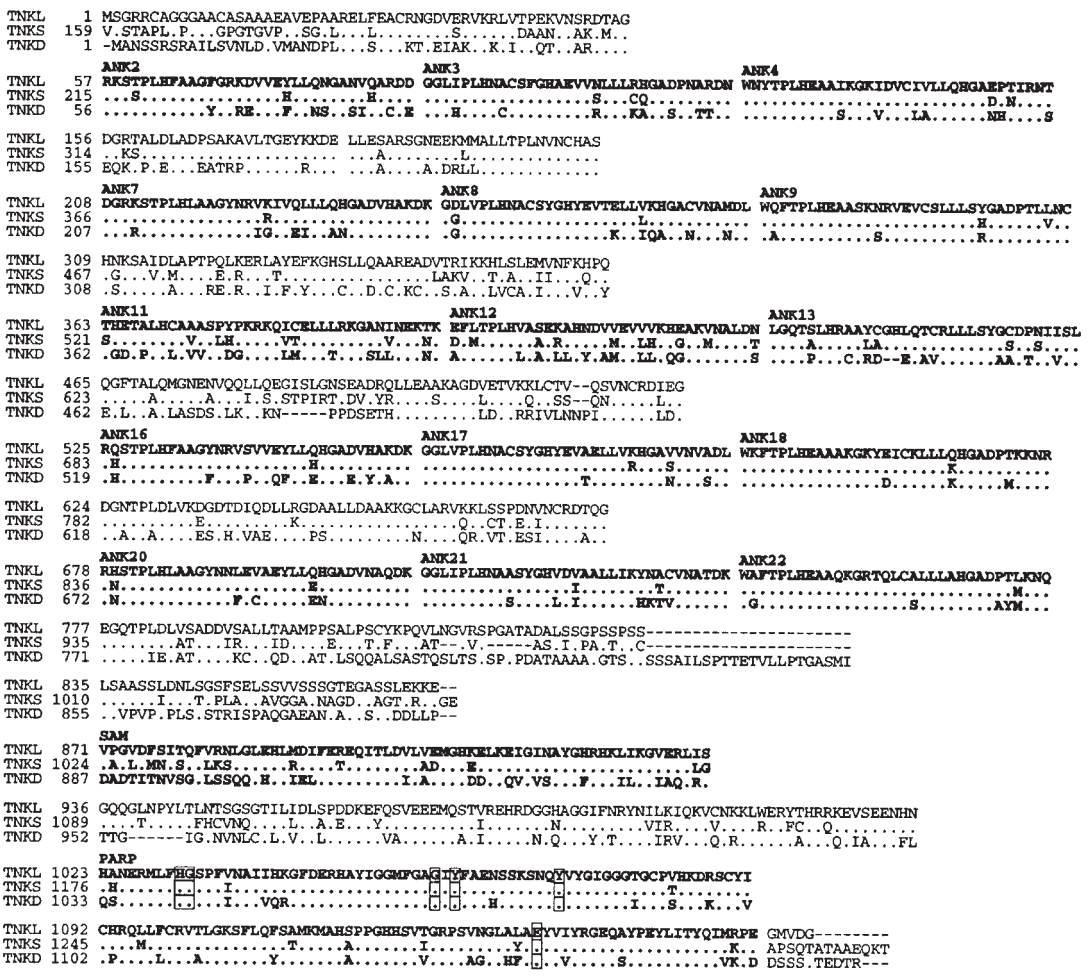

Figure 1 Multiple alignment of predicted amino acid sequences of tankyrase protein family: TNKL (GenBank accession number AF264912), tankyrase (TNKS, GenPept accession number AAC79841), and their ortholog from *Drosophila melanogaster* (TNKD, GenPept accession number AAD34784). The alignment was produced using ClustalW algorithm,[12,13] an interface of BCM Search Launcher (http://www.hgsc.bcm.tmc.edu/SearchLauncher/).[14] To analyze the domain structure, the amino acid sequences were run against database of PROSITE profiles using ProfileScan[15] server (http://www.isrec.isb-sib.ch/software/PFSCAN_form.html) with a cut-off normalized match score of 6.5 ('include weak matches'), and against Pfam database http://pfam.wustl.edu/). Dashes indicate gaps, and dots replace conservative amino acid residues identical to those in TNKL sequence. For easy comparison, the numbering of ANK domains is the same as in the original report on tankyrase.[3] The homology domain with sterile alpha motif, and C-terminal PARP catalytic domain are designated above the sequences. Boxed residues are conserved in human and *D. melanogaster* PARPs as well as in prokaryotic ADP-ribosyltransferases, exotoxin A from *P. aeruginosa* and diphtheria toxin.

in the presence of [^{32}P]NAD^{+} under experimental conditions described by Smith *et al*[3] (data not shown). Therefore, TNKL might be involved in the posttranslational modification of itself and of unidentified substrate(s) as part of yet unknown signaling pathway(s).

The SAM motif (77% amino acid identity with human tankyrase) and ankyrin repeat region (85% amino acid identity with tankyrase) are thought to function in protein–protein interactions. Most of the ankyrin repeat region is also homologous to human ankyrin 1 (GenPept accession number AAB47805), for which a common ancestry with tankyrase was suggested.[4] Based on these findings, we designated the gene product encoded by MO-BC-203 as TNKL (tankyrase-like protein).

The cloned cDNA insert of MO-BC-203 lacked the initiation codon and appeared truncated at ankyrin repeat 8 (see Figure 2) according to the numbering in tankyrase.[3] Additional cDNA sequence was isolated from the same MO-BC phage library using a MO-BC-203 insert as a probe. Clone I203, with an insert of 3403 bp appeared to be truncated at both 5′ and 3′ termini (Figure 2), but allowed to extend the cDNA sequence to ankyrin repeat 2. The rest of the 5′ terminus of TNKL cDNA was defined as described below.

The nucleotide sequence of TNKL was complementary to STS sequences stSG15491, WI-16054, and WI-8339, and many expressed sequence tags (ESTs) grouped in UniGene build 120 (http://www.ncbi.nlm.nih.gov/UniGene) cluster Hs.280776, described as 'ESTs, moderately similar to TRF1- interacting ankyrin-related ADP-ribose polymerase [H.sapiens]'. This cluster was mapped to chromosome 10q23–10q24, while the tankyrase gene was previously mapped to chromosome 8.[4] Therefore, the genes encoding the tankyrase and *tnkl* are distinct and together with their *D. melanogaster* homologue may form a novel gene family.

The sequence tagged sites (STSs) described above as well as the entire sequence of clones MO-BC-203 and I203

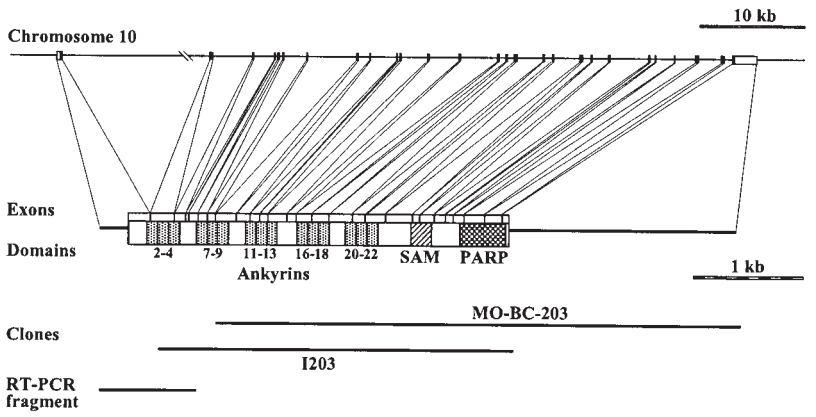

Figure 2 Exon-intron structure of *tnkl* gene and domain structure of predicted TNKL protein. Boxes indicate parts of encoded protein defined as described in legend to Figure 1: Ankyrins (ANK), conservative ankyrin repeats (shaded); SAM, homology to sterile alpha motif (stripped); PARP, PARP-like catalytic domain (patterned). The numbering of ANK domains follows that of Smith *et al*.[3] Lines below represent the original cDNA clone MO-BC-203 identified using SEREX; an additional clone I203 found by hybridization with radioactive DNA probe; and the 5′-terminal region defined by direct sequencing of 0.9 kb RT-PCR product prepared using 200 ng of human testis polyA$^+$ RNA. The oligonucleotides to prime the reaction were designed using unfinished genome sequence RP11–251A15 (GenBank accession number AL359707) as follows: ATC TAG ACC GCG TCG TTT CAG GAC CC (plus) and GTT GAC TTT CTG CCA TCA CTT GCG TG (minus). The first cDNA strain was synthesized with RevertAid™ M-MuLV Reverse Transcriptase (Fermentas, Vilnius, Lithuania), and then cDNA amplification was reformed with Advantage™-GC PCR kit (Clontech, Palo Alto, CA, USA). The 0.9-kb product was excised out of agarose gel and sequenced using nested primer AAA GGA GAT TGA CTA CTT CAG C (minus strand).

were complementary to recently deposited sequences of BAC clones from human chromosome 10: RP11–251A15 (Genbank accession number AL359707) and RP11–402D21 (Genbank accession number AL359198), which appear to contain exons of the *tnkl* gene. The putative exon 1 was found using the tblastn software (http://www.ncbi.nlm.nih.gov/BLAST/) as a sequence within RP11–251A15 with homology to the 5′-ends of cDNAs encoding human tankyrase and tankyrase-like protein of *D. melanogaster*. The existence of transcripts originating from this putative exon and the position of the splicing site were confirmed by direct sequencing of a reverse transcription-polymerase chain reaction (RT-PCR) fragment using human testis polyA$^+$ RNA as a template (Figure 2 and data not shown).

The predicted full-length TNKL protein contains 1166 amino acids, with molecular weight 127 kDa in non-modified form. Overall, the predicted amino acid sequence of TNKL is 78% identical to human tankyrase and 62% identical to putative tankyrase-like protein of *D. melanogaster*. Based on the homologies between the full-length cDNA and genomic sequences it appears that the human *tnkl* gene spans more than 60 kb and contains at least 27 exons (Figure 2).

Our analysis of the TNKL and tankyrase protein sequences using the PROSITE and Pfam databases of profiles revealed only 15 ankyrin repeats in homologous positions for each of the two proteins (see Figures 1 and 2), with normalized match scores ranging from 10 to 15 for TNKL, from 11 to 16 for tankyrase and from 7 to 15 for the *D. melanogaster* ortholog. Both human tankyrase and TNKL contain five groups of three ANK units each (Figures 1 and 2). Although spacer regions do contain some weak similarities to ankyrin, no additional ANK units could be assigned with confidence.

Distinct features of the TNKL amino acid sequence include the N-terminal domain which appears to lack homology to tankyrase, and the high degree of sequence divergence between the three tankyrase-like proteins observed over the ankyrin-like repeat number 24 (as defined by Smith *et al*.[3] Like tankyrase, TNKL lacks a detectable nuclear localization sequence, so its subcellular distribution might be defined by its interacting protein partners.

Since TNKL (MO-BC203) shows cancer-related serological profile (manuscript in preparation), tissue-specificity of its expression was addressed. Northern blot analysis revealed a single major transcript of approximately 7 kb expressed in all tested normal adult and fetal tissues (Figure 3a, lanes 1–17, and data not shown). The apparent 7 kb size of *tnkl* transcript corresponds to the sequenced cDNA with coding part of 3.5 kb, 3′ untranslated region of 2.5 kb, and 5′ untranslated region of 1 kb. Therefore, in contrast to many other cancer antigens isolated by SEREX, normal expression of TNKL is not restricted to testis or any other tissue, at least at the level of transcription. Moreover, there was no significant change in the level of the *tnkl* transcript in the MO-BC tumor (Figure 3a, lane 19), arguing against overexpression at the transcriptional level. Additionally, Southern analysis of DNA isolated from several tumor specimens failed to provide evidence for possible *tnkl* gene amplification in neoplasia (Figure 3b). These findings suggest that the immune response against TNKL in cancer may result from mutation, post-synthetic modification, or cancer-related breakdown of tolerance. We are currently generating antibodies to address the pattern of expression of TNKL in normal and cancer tissues at the protein level.

The microsatellite marker D10S583 located close to the *tnkl* locus was recently shown to lose heterozygosity in 32% of breast tumors.[5] The distance between D10S583 and known STS markers within the *tnkl* gene can be estimated based both on the radiation hybrid-based GeneMap'99 and on an incomplete physical map of human chromosome 10 from the Sanger Centre (http://webace.sanger.ac.uk/); it appears not to exceed 3 cM. Future analysis will reveal whether the reported loss of hetero-

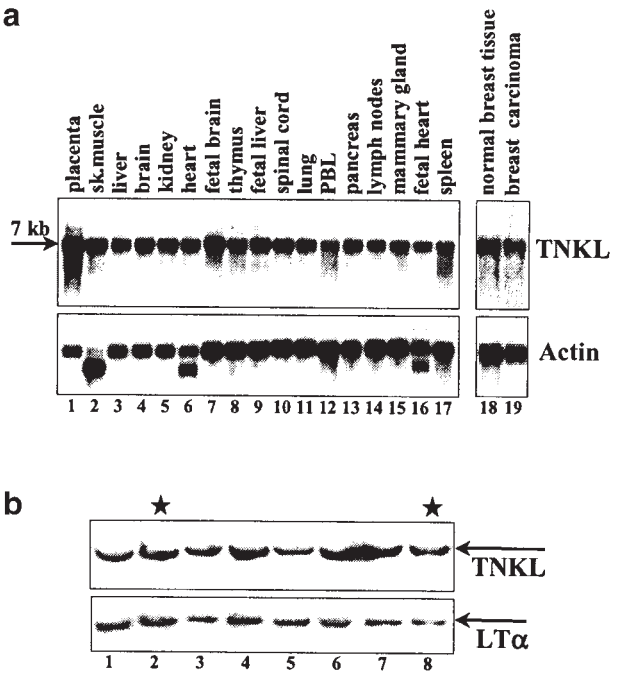

Figure 3 Northern (**a**) and Southern (**b**) blot analysis of TNKL expression. A. For Northern analysis 1 μg of polyA$^+$ RNA (Clontech, Paolo Alto, CA, USA) of each human tissue was used for electrophoresis and blotting onto Hybond N$^+$ membrane (Amersham, Uppsala, Sweden). The membrane was hybridized to TNKL probe according to manufacturer's instruction, exposed to X-ray film, washed and rehybridized with actin probe. The right panel shows a lack of TNKL overexpression in the sample of MO-BC tumor used for cloning of TNKL. B. For Southern analysis 10 μg of DNA from normal breast tissue (left lane) and seven breast tumors were digested with EcoR1, loaded onto agarose gel, run, transferred to Hybond N$^+$ membrane, hybridized to TNKL probe, exposed to X-ray film, washed and rehybridized with human lymphotoxin alpha probe. Stars indicate samples from patients positive for antibodies against TNKL. All tumor specimens were obtained from the Oncological Research Center, Russian Academy of Medical Sciences (Moscow, Russia).

zygosity in the 10q23–10q24 region in cancers[5–8] may be associated with mutations or truncations in *tnkl* in tumors.

The physiological function of TNKL, its substrates and other protein partners remain unknown. Its homologue, human tankyrase, is thought to be involved in telomere maintenance.[3] Recently, the reverse transcriptase of human telomerase (hTERT) was identified as a tumor associated antigen capable of inducing CTL responses.[9] On the other hand, a cytoplasmic localization of the bulk of tankyrase molecules[10] may suggest additional functions. After submission of this manuscript we learned the results of Chi and Lodish[11] who demonstrated that tankyrase is phosphorylated by MAP-kinase cascades and is targeted at IRAP, a Golgi-associated protein expressed in muscle cells and adipocytes. If this similarity extends to TNKL, then abnormal levels of modification may contribute to immune response in cancer patients, since the MAP-kinase cascades may be affected in cancer cells.

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