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Advances in Brief

Characterization of Alkaline Phosphatase Genes Expressed in Seminoma by cDNA Cloning

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Abstract

Two members of a placental alkaline phosphatase (PLAP) family, PLAP and PLAP-like or germ cell alkaline phosphatase, are aberrantly expressed in tumors of ecotropic origin. To characterize alkaline phosphatase induced in seminoma, alkaline phosphatase cDNA clones were isolated from a cDNA library constructed from seminoma cells and characterized by nucleotide sequence determination. Thus, isolated cDNA clones were classified into two types, germ cell alkaline phosphatase (PLAP-like) and liver/bone/kidney-type alkaline phosphatase (L/B/K AP). These results suggest that other than the PLAP family members, the expression of L/B/K AP is enhanced in seminoma and can serve as a tumor marker in seminoma.

Introduction

Human APs[2] can be classified into at least five tissue-specific forms or isozymes mainly according to the specificity of the tissue to be expressed, termed PLAP (or Regan isozyme), IAP, L/B/K AP, germ cell AP (PLAP-like or NAGAO isozyme), and IAP-like (Kasahara isozyme; Ref. 1). AP is present in nearly all living organisms, with the exception of some higher plants, but little is known regarding the physiological function of AP in most tissues, except that the bone isozyme (L/B/K AP) is supposed to have a role in normal skeletal mineralization (2); a missense mutation in the *L/B/K AP* gene is responsible for the development of hypophosphatasia (3).

Genes encoding two members of a human PLAP family, *PLAP* and germ cell AP (*PLAP-like*), are located close to each other on the long arm of chromosome 2 (4, 5), forming a multigene family along with the *IAP* gene, probably brought about as a result of gene duplication. In fact, these *PLAP, PLAP-like,* and *IAP* genes are more closely related to each other than other *AP* genes (6, 7). Furthermore, the *PLAP* and *PLAP-like* genes are quite polymorphic, with three common variants and at least 15 rare alleles at the *PLAP* locus (5, 8) and with two alleles at the *PLAP-like* locus (9), in contrast to the complete lack of genetic variation in other *AP* genes. This extensive genetic diversity recognized in the *PLAP* genes may be possibly related to their particular function in the placental or other unknown tissues.

The expression of the two human placental isozymes of AP is usually confined to the syncitiotrophoblast from 12 weeks of pregnancy to term, and testis and thymus, respectively. However, interestingly enough, they are reexpressed in various malignant tumors including bronchogenic carcinoma (10), pleuritis carcinomatosa (11), gynecological cancers (12), and seminoma (13). In particular, remarkable enhancement of the germ cell AP expression was observed in the sera and biopsy tissues of patients with germ cell tumors of the testis, particularly seminoma (14, 15). Thus, the induction of the germ cell *AP* gene has been suggested, not only to be a clinically useful serological tumor marker for early detection of the seminoma-type testicular cancer but also to provide a clue to develop an effective cure for this cancer. However, APs enhanced for their activity in seminoma have not been fully characterized yet. In fact, we have recently purified a novel type of AP that could be distinguished from the known APs in terms of molecular weight calibrated by gel filtration, the response to various inhibitors such as L-phenylalanine and L-leucine, and its immunological reaction.[3] In this study, to investigate more about APs induced in seminoma, a cDNA library was constructed from testicular cancer cell clones with seminoma, and cDNA clones for APs isolated from it were characterized.

Materials and Methods

Construction of a Seminoma cDNA Library. A seminoma type of testicular cancer cells at stage IIB was obtained from a 24-year-old Japanese man. Total RNA was extracted from them by the homogenate procedure in the guanidine thiocyanate solution followed by CsCl centrifugation at 36,000 rpm and 20°C for 12 h. Poly(A)-enriched RNA was purified by oligo-deoxythymidylate cellulose chromatography. A seminoma cDNA library was constructed from this poly(A)-enriched RNA using the ZAP-cDNA synthesis kit according to the protocol provided by the manufacturer (Stratagene, La Jolla, CA). Briefly, the first-strand cDNA was synthesized by priming of Moloney murine leukemia virus reverse transcriptase reactions with oligo(dT) linker-primer including a *Xho*I recognition site using 5-methyl dCTP, which protects the cDNA from restriction enzyme used in subsequent cloning step. The product of first-strand synthesis, a cDNA-mRNA hybrid, was used as a template for nick-translation reaction with RNase H. This reaction created a series of RNA primers that was used by *Escherichia coli* DNA polymerase I to produce the second strand of cDNA. The uneven termini of the resultant cDNA species were filled in with Pfu polymerase, and *Eco*RI adapters were ligated to the blunt ends. Then, the *Xho*I-digested and size-fractionated cDNA was ligated to the Uni-ZAP XR vector arms at the *Xho*I-*Eco*RI sites. This library consisted of $\sim 1 \times 10^5$ independent recombinant phages.

Probe for Screening of a cDNA Library. Two PCR-amplified products containing portions of the germ cell *AP* gene (*PLAP-like*) were used for screening of the cDNA library as probes. A 1051-bp fragment encompassing the segment from the midst of exon 3 to exon 6 in the germ cell *AP* gene (from nucleotide positions 903 to 1953; Ref. 14) was amplified using the following primers: primer 1 (forward), 5′-GCCTGAGACCTTCCTGGCCAT-3′; and primer 2 (reverse), 5′-CTGGTGCTTCGCCAGCCATTC-3′. Another 653-bp fragment encompassing the segment from the midst of exon 9 to the midst of exon 11 (from nucleotide positions 2616 to 3268; Ref. 14) was amplified using the following primers: primer 3 (forward), 5′-GTCTTCTCCTTCGGAGGCTAC-3′; and primer 4 (reverse), 5′-GAAGGCCATGACGTGCGCTAT-3′. PCR reactions were carried out using 100–200 ng of high molecular mass human genomic DNA of Japanese origin with the 5 mM PCR primer pair in a standard 25-ml reaction containing 1.2 units of TAKARA LA Taq polymerase (TaKaRa, Ohtu, Shiga, Japan), 0.2 mM deoxynucleotide triphosphate, 25 mM $MgCl_2$, and LA PCR buffer (TaKaRa). Reaction mixture was placed in a

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[2] The abbreviations used are: AP, alkaline phosphatase; PLAP, placental AP; L/B/K AP, liver/bone/kidney-type AP; IAP, intestinal AP.

[3] T. Yamamoto and Y. Katsuoka, unpublished data.

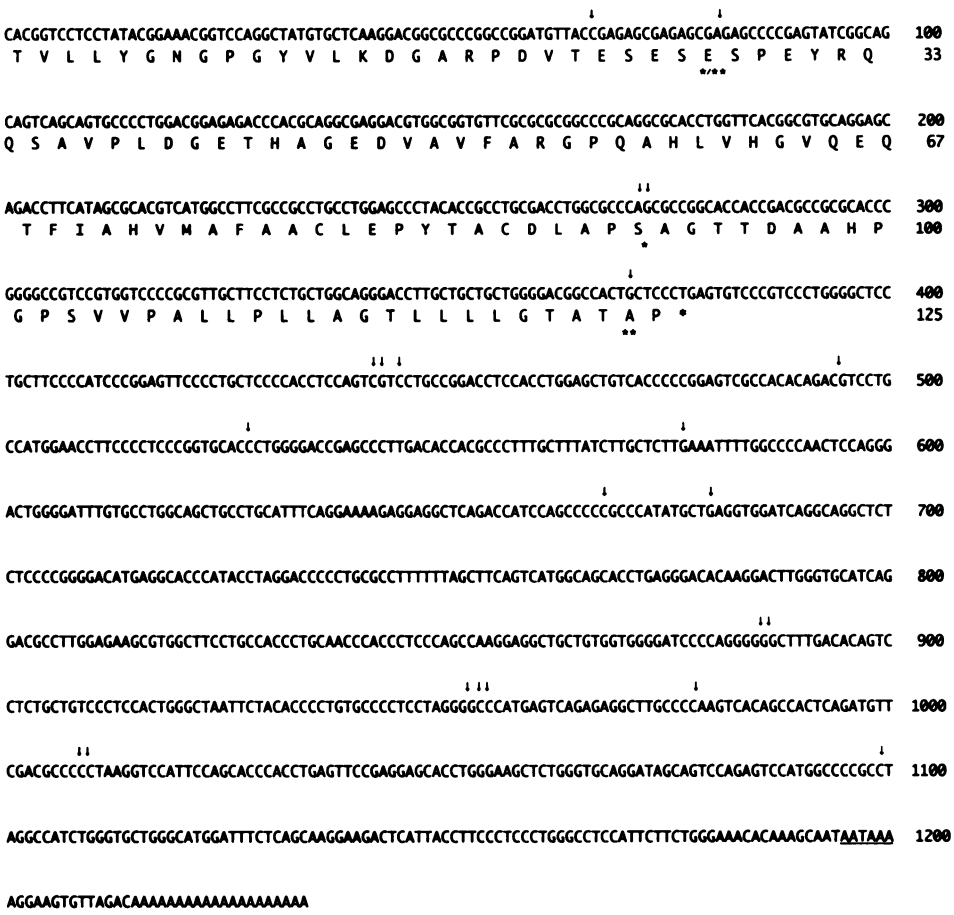

Fig. 1. Nucleotide and deduced amino acid sequences of the cDNA clones derived from seminoma, p5-2 encoding AP. *Arrows*, 22 nucleotide substitutions between p5-2 and one variant of germ cell AP reported previously by Millan and Manes (14). *, two amino acid substitutions, Glu for Gly at residue 434 and Ser for Pro at residue 498 between p5-2 and one variant of germ cell AP reported previously by Millan and Manes (14). **, two amino acid substitutions, Glu for Gly at residue 434 and Ala for Thr at residue 531 between p5-2 and another variant of germ cell AP reported previously by Gum *et al.* (9). A putative poly(A) additional signal AATAAA is *underlined*. This cDNA clone represents a transcript from a new allele of the germ cell AP gene.

thermal cycler (GeneAmp PCR system 9600; Perkin-Elmer, Piscataway, NJ) and subjected to denaturation at 94°C for 2 min, followed immediately by 30 cycles of denaturation at 98°C for 10 s and annealing and elongation at 68°C or 2 min. The identities of both of the PCR-amplified products were confirmed by direct nucleotide sequencing, which was carried out by the cycle sequencing method using AmpliTaq DNA polymerase FS (PE Applied Biosystems, Piscataway, NJ), fluorescently labeled dye terminators in a Gene Amp PCR system (PE Applied Biosystems), and an ABI 373S DNA sequencing machine.

Library Screening. The seminoma cDNA Library was screened for clones specifying AP, using plaque hybridization under the less stringent conditions. Approximately 1×10^5 phages were screened, using the PCR-amplified gene probes radiolabeled by *in vitro* DNA synthesis with the Klenow fragment of *E. coli* DNA polymerase I.

Nucleotide Sequence[4] Determination of cDNA Clones. The cDNA inserts were excised from the phage vector to the pBluscript phagemid using ExAssist helper phage *in vivo*. Phagemid DNA was prepared and sequenced by the dye primer method using fluorescently labeled dye primers and an ABI 373S DNA sequencing machine. If necessary to fill the sequence gaps, PCR products amplified by appropriate primers were subcloned by the TA cloning kit (Invitrogen, San Diego, CA).

Results and Discussion

To isolate cDNA clones encoding APs induced in seminoma, a cDNA library was constructed from poly(A)-enriched RNA prepared from a seminoma type of testicular cancer cells. This cDNA library was screened by plaque hybridization with the two genomic probes, obtained by PCR amplification from exons 3 to 6 and from exons 9 to 11 in the germ cell *AP* gene (*PLAP-like*; Ref. 14). Theses two segments, which contain the residues that interact with substrate phosphate (exons 4 and 5) and metal ligand sites (exons 5 and 9), are well conserved among the five *AP* genes (*PLAP, IAP, L/B/K AP, PLAP-like,* and *IAP-like*) thus far cloned. The exon 9–11 segment of the germ cell *AP* gene displays more than 98 and 90% identity at the nucleotide level with the *PLAP* and *IAP* genes, and more than 70% with the most distantly related gene, *L/B/K AP*. Such high nucleotide identity in these segments possibly allows the probes used for screening of the library to pick up cDNA clones for any type of the APs, if they were expressed in seminoma.

In total, eight cDNA clones among 1×10^5 plaques were thus isolated and constructed for their restriction maps using representative six-base recognition restriction enzymes. Although any of them did not seem to represent full-length transcripts, these cDNA clones could be classified into two groups (three and five clones) in this way. Two clones, p5-2 and p15-2, carrying the longest inserts were chosen among the former and latter groups, respectively, and subjected to nucleotide sequence determination.

The cDNA insert of p5-2 consists of 1234 bp, including most of the one-third of the transcript, 375 bp of the 3′-terminal coding region (125 residues from the COOH-terminal end), and the entire 3′ untranslated region (Fig. 1). The p5-2 cDNA nucleotide sequence reveals 98.2% identity (22 nucleotide differences) in a 1230 nucleotide overlap with the germ cell *AP* gene (14). Furthermore, the deduced amino acid sequence of p5-2 reveals 98.4% amino acid identity in a 125-amino acid overlap with germ cell AP. There are only two amino acid substitutions, Glu for Gly at residue 434 and Ser for Pro at residue 498 between p5-2 and one variant of germ cell AP reported by Millan and Manes (14). Between p5-2 and another variant of germ cell AP reported by Gum *et al.* (9), there are also two amino acid

[4] The nucleotide sequence data reported in this study will appear in DNA Data Bank of Japan, European Molecular Biology Laboratory and GenBank nucleotide sequence databases with the following accession numbers: AB012642 and AB012643.

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GGGACTGGTACTCAGACAACGAGATGCCCCCTGAGGCCTTGAGCCAGGGCTGTAAGGACATCGCCTACCAGCTCATGCATAACATCAGGGACATTGACGT      100
  D  W  Y  S  D  N  E  M  P  P  E  A  L  S  Q  G  C  K  D  I  A  Y  Q  L  M  H  N  I  R  D  I  D  V       33

GATCATGGGGGGTGGCCGGAAATACATGTACCCCAAGAATAAAACTGATGTGGAGTATGAGAGTGACGAGAAAGCCAGGGGCACGAGGCTGGACGGCCTG      200
  I  M  G  G  G  R  K  Y  M  Y  P  K  N  K  T  D  V  E  Y  E  S  D  E  K  A  R  G  T  R  L  D  G  L       66

GACCTCGTTGACACCTGGAAGAGCTTCAAACCGAGACACAAGCACTCCCACTTCATCTGGAACCGCACGGAACTCCTGACCCTTGACCCCCACAATGTGG      300
  D  L  V  D  T  W  K  S  F  K  P  R  H  K  H  S  H  F  I  W  N  R  T  E  L  L  T  L  D  P  H  N  V  D      100

ACTACCTATTGGGTCTCTTCGAGCCGGGGGACATGCAGTACGAGCTGAACAGGAACAACGTGACGGACCCGTCACTCTCCGAGATGGTGGTGGTGGCCAT      400
  Y  L  L  G  L  F  E  P  G  D  M  Q  Y  E  L  N  R  N  N  V  T  D  P  S  L  S  E  M  V  V  V  A  I      133

CCAGATCCTGCGGAAGAACCCCAAAGGCTTCTTCTTGCTGGTGGAAGGAGGCAGAATTGACCACGGGCACCATGAAGGAAAAGCCAAGCAGGCCCTGCAT      500
  Q  I  L  R  K  N  P  K  G  F  F  L  L  V  E  G  G  R  I  D  H  G  H  H  E  G  K  A  K  Q  A  L  H      166

GAGGCGGTGGAGATGGACCGGGCCATCGGGCAGGCAGGCAGCTTGACCTCCTCGGAAGACACTCTGACCGTGGTCACTGCGGACCATTCCCACGTCTTCA      600
  E  A  V  E  M  D  R  A  I  G  Q  A  G  S  L  T  S  S  E  D  T  L  T  V  V  T  A  D  H  S  H  V  F  T      200

CATTTGGTGGATACACCCCCCGTGGCAACTCTATCTTTGGTCTGGCCCCCATGCTGAGTGACACAGACAAGAAGCCCTTCACTGCCATCCTGTATGGCAA      700
  F  G  G  Y  T  P  R  G  N  S  I  F  G  L  A  P  M  L  S  D  T  D  K  K  P  F  T  A  I  L  Y  G  N      233

TGGGCCTGGCTACAAGGTGGTGGGCGGTGAACGAGAGAATGTCTCCATGGTGGACTATGCTCACAACAACTACCAGGCGCAGTCTGCTGTGCCCCTGCGC      800
  G  P  G  Y  K  V  V  G  G  E  R  E  N  V  S  M  V  D  Y  A  H  N  N  Y  Q  A  Q  S  A  V  P  L  R      266

CACGAGACCCACGGCGGGGAGGACGTGGCCGTCTTCTCCAAGGGCCCCATGGCGCACCTGCTGCACGGCGTCCACGAGCAGAACTACGTCCCCCACGTGA      900
  H  E  T  H  G  G  E  D  V  A  V  F  S  K  G  P  M  A  H  L  L  H  G  V  H  E  Q  N  Y  V  P  H  V  M      300

TGGCGTATGCAGCCTGCATCGGGGCCAACCTCGGCCACTGTGCTCCTGCCAGCTCGGCAGGCAGCCTTGCTGCAGGCCCCCTGCTGCTCGCGCTGGCCCT     1000
  A  Y  A  A  C  I  G  A  N  L  G  H  C  A  P  A  S  S  A  G  S  L  A  A  G  P  L  L  L  A  L  A  L      333

CTACCCCCTGAGCGTCCTGTTCTGAGGGCCCAGGGCCCGGGCACCCACAAGCCCGTGACAGATGCCAACTTCCCACACGGCAGCCCCCCCCTCAAGGGGC     1100
  Y  P  L  S  V  L  F  *                                                                                  340

AGGGAGGTGGGGGCCTCCTCAGCCTCTGCAACTGCAAGAAAGGGGACCCAGGAAACCAAAGTCTGCCGCCCACCTCGCTCCCCTCTGGAATCTTCCCCAA     1200

GGGCCAAACCCACTTCTGGCCTCCAGCCTTTGCTCCCTCCCCGCTGCCCTTTGGCCAACAGGGTAGATTTCTCTTGGGCAGGCAGAGAGTACAGACTGCA     1300

GACATTCTCAAAGCCTCTTA TTTTTCTAGCGAACGTATTTCTCCAGACCCAGAGGCCCTGAAGCCTCCGTGGAACATTCTGGATCTGACCCTCCCAGTCT     1400

CATCTCCTGACCCTCCCACTCCCATCTCCTTACCTCTGGAACCCCCCAGGCCCTACAGTGCTCATGTCCCTGTCCCCAGGCCCAGCCCTCCTTCAGGGGA     1500

GTTGAGGTCTTTCTCCTCAGGACAAGGCCTTGCTCACTCACTCACTCCAAGACCACCAGGGTCCCAGGAAGCCGGTGCCTGGGTGGCCATCCTACCCAGC     1600

GTGGCCCAGGCCGGGAAGAGCCACCTGGCAGGGCTCACACTCCTGGGCTCTGAACACACACGCCAGCTCCTCTCTGAAGCGACTCTCCTGTTTGGAACGG     1700

CAAAAAAAAATTTTTTTTTTCTCTTTTTGGTGGTGGTTAAAAGGGAACACAAAACATTTAAATAAAACTTTCCAAATATAAAAAAAAAAAAAAAAAA      1798
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Fig. 2. Nucleotide and deduced amino acid sequences of the cDNA clones derived from seminoma, p15-2 encoding AP. A putative poly(A) additional signal AATAAA is *underlined*. This cDNA clone represents a transcript from the *L/B/K AP* gene*, translational termination codon.

substitutions, Glu for Gly at residue 434 and Ala for Thr at residue 531 (Fig. 1). These results suggest that p5-2 represents a transcript from a new allele of the germ cell *AP* gene.

The cDNA insert of the other clone, p15-2, consists of 1799 bp including the almost two-thirds of the transcript, 1023 bp of the 3′-terminal coding region (341 residues from the COOH-terminal end) and the entire 3′ untranslated region (Fig. 2). The p15-2 cDNA nucleotide sequence reveals complete identity in a 1390-nucleotide overlap with the L/B/K AP cDNA clone reported by Kishi *et al.* (16), although their cDNA clone is apparently lacking the most 3′ end segment, including a poly(A) tail. p15-2 also shows high nucleotide identity (98.5%) in a 1033-nucleotide overlap with another L/B/K AP cDNA clone with a different allele (7). Between them, there is only one amino acid substitution, His for Tyr at residue 263. These results clearly demonstrate that p15-2 represents a transcript from the *L/B/K AP* gene.

Despite an apparent association between the induction of AP and various cancers (10–13), no distinct differences between testicular tumor-derived AP and thus far known APs such as PLAP have been recognized (14, 15). In this study, eight cDNA clones for AP were isolated from the seminoma cDNA library. Among them, three and five clones represent transcripts from the *PLAP-like* and *L/B/K AP* genes, respectively. The number of the cDNA clones isolated and characterized in this study was limited, and these cDNA analyses were qualitative rather than quantitative. However, these results suggest that germ cell AP and L/B/K AP are two major species strongly reexpressed in seminoma. In addition, both of germ cell AP and L/B/K AP could be detected in seminoma by biochemical analyses such as calibration of molecular weight and enzyme assay in the presence of various inhibitors, confirming the expression of these two *PLAP-like* and *L/B/K AP* genes in seminoma. To our knowledge, our study here is the first finding of up-regulation of L/B/K AP in

seminoma. Thus, the expression of L/B/K AP as well as germ cell AP can serve as a tumor marker in seminoma. The expression of L/B/K AP enzyme activity is under the control of various stimuli at the transcriptional level (17–19). In particular, two different mRNAs produced by alternative splicing or alternative transcription initiation in the mouse or rat, respectively, appear to be strictly regulated for the tissue-specific expression in response to several hormones and other regulators (19, 20). The induction of L/B/K AP associated with tumorization such as in seminoma might result from the involvement of the same regulatory factors controlling such expression of L/B/K AP enzyme activity in various tissues, which remains to be determined.

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