Immunology and Biochemistry of the Regan Isoenzyme

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The Regan isoenzyme is a placental-type alkaline phosphatase that is expressed in a number of human tumors, particularly in gonadal and urologic cancers. Attention is given to the unique gene that codes for placental alkaline phosphatase and the similarities and differences in the tumor and placental gene products. The separation and identification of individual organ-specific isoenzymes is accomplished by a variety of biochemical, immunologic, and electrophoretic techniques and the correlation of the Regan isoenzyme, non-Regan isoenzyme, and Nagao isoenzyme, and the Kasahara isoenzyme is made with their developmental counterparts. The L-leucine-sensitive phenotypes of placental and tumor alkaline phosphatases and the non-Regan early placental type alkaline phosphatases appear to be developmental phase-specific. Oncotrophoblast gene expression has been investigated with monophenotypic cell culture lines as a consequence of modulation by prednisolone and hyperosmolarity. Finally, general discussion of oncodevelopmental proteins as tumor markers precedes a current opinion of Regan isoenzyme as a tumor marker. Evidence now points to seminoma as a consistent producer of Regan isoenzyme although much more work will be required to establish its clinical utility.

Key words: oncodevelopmental protein, Regan isoenzyme, Nagao isoenzyme, Kasahara isoenzyme, prednisolone, tumor markers

INTRODUCTION

To the medical world, one of the most commonly requisitioned laboratory tests is the serum alkaline phosphatase. It was introduced by people like H.D. Kay and Earl King at the University of Toronto in the early 30s and extended considerably in physiologic and pathologic studies by the Bodansky brothers and by the Gutmans later. The test has its place in modern medicine in relation to the differential diagnosis and management diseases of liver and bone.

In fact, in the ultramodern day of tumor markers, serum alkaline phosphatase elevations have been consistently associated with osteogenic sarcoma since the days of the pioneer workers. It has served well in alerting the physician to the presence of this tumor and in monitoring the patient under therapy for the status of the sarcoma. Cultures of osteogenic sarcoma produce high levels of alkaline phosphatase, but of two types which will be discussed later.

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The historic event that presaged the study of enzymes as tumor markers was the introduction by Markert and Hunter of electrophoresis as a means of separating the members of a family of proteins sharing the same catalytic activity—in other words, isoymes. The most attractive model at that time was lactate dehydrogenase-LDH, which separated beautifully into five bands, explainable as representing combinations of like or unlike monomeric molecules.

The application of electrophoresis on starch gel and later on polyacrylamide gels to tissue and serum alkaline phosphatases produced a bewildering number of isozyme bands from extracts of tissue and from serum. Their tissue origin could be guessed only by their position on the gel. We have looked for biochemical and immunologic characteristics for their identification.

At this time, I would like to share with you the organization of this article. The title “Immunochemistry and Biochemistry of the Regan Isoenzyme” should be reversed because the biochemistry came first and the immunoochemistry second. I will describe 1) how each technology and their combinations proved necessary to sort out the families of alkaline phosphatases, 2) how the Regan isoenzyme (a form of placental alkaline phosphatase) was discovered, 3) what the other oncodevelopmental alkaline phosphatases are, 4) how model cell lines can be used to study modulation of gene expression, 5) what is known of the developmental biology of placental alkaline phosphatase, and 6) where clinical interest is now centered with regard to the oncotrophoblast alkaline phosphatases.

METHODS

Organ-Specific Isoenzyme Inhibition

In 1963 it was discovered that L-phenylalanine but not the D-isomer was an inhibitor of intestinal alkaline phosphatase but not liver or bone types [1]. Four years later we demonstrated that L-phenylalanine also inhibits placental alkaline phosphatase [2]. We also discovered that L-homoarginine had just the opposite specificity, inhibiting the isozymes of liver and bone [3]. New peptide inhibitors [4] and carbohydrate inhibitors [5] have been recently introduced.

Physical Properties of the Isoenzymes

Moss and King [6] reported the remarkable heat lability of bone alkaline phosphatase and Posen and his co-workers [7] found that placental alkaline phosphatase was very heat-stable.

Immunologic Techniques

Specific antisera prepared in rabbits have proved useful as immunologic reagents for distinguishing between placental, intestinal, and liver type isozymes. They have found their greatest use in antigen retardation electrophoretic tests [8]. Radioimmunoassays have been published for measuring Regan isoenzyme [9,10] and intestinal isoenzyme [11] in serum but have not gained wide acceptance.
Electrophoretic Techniques

Aside from the widely accepted electrophoretic methods using supporting matrices of either starch, polyacrylamide and cellulose acetate, it is necessary at times to use a combination of amino acid inhibition, heat inactivation, and antigen retardation techniques to identify the organ source of the isoenzyme.

Quantitation of Individual Isoenzymes in Mixtures

The principle of differential L-phenylalanine inhibition on unheated serum and heated serum (15 min at 56°C) permitted through the use of nomograms an estimate of intestine, liver, and bone isoenzymes [12-14].

More recently, these methods have been improved and miniaturized in a paper by Millan and co-workers [15] dealing with the isozyme expression in hypophosphatasia.

The placental isoenzyme could be measured directly on serum heated for 5 minutes at 65°C. Its identity is readily confirmed with the antigen retardation test using a fluorogenic substrate [8].

The Regan Isoenzyme

This isoenzyme of alkaline phosphatase was discovered in the serum, primary lung cancer tissue, and in a number of metastatic tissues as well [18]. The progressive terminal rise in serum alkaline phosphatase was unaccompanied by any change in SGOT, SGPT, and bilirubin levels or positive bone scan. Approximately 50% of the elevated serum alkaline phosphatase was heat-stable and L-phenylalanine-sensitive. These properties could be attributed to a single isozyme band which on starch gel electrophoresis before and after neuraminidase treatment was indistinguishable from placental alkaline phosphatase.

Our immunologic studies [19] demonstrated identity of placental and tumor alkaline phosphatase antigens when tested against the corresponding antisera in Ouchterlony plates. Also, equivalent losses in enzyme activity of the enzyme-antibody complexes were observed and similar specific retardation of the enzyme-antibody complex was noted.

Terminology

At this point, it is important to note that the isoenzyme was named after the first patient found to express it and it was not designated placental alkaline phosphatase. The latter exists in over 40 distinct allelic forms as evidenced by electrophoresis. Unless one can produce evidence of complete amino acid homology between tumor isoenzyme and one placental isoenzyme protein, its exact identity remains unproved. However, one can legitimately refer to Regan isoenzyme as placental-type, in the meanwhile.

Reference should also be made to the reasons for introducing the term "oncodevelopmental protein" in describing the Regan isoenzyme. The prior discoveries of alpha-fetoprotein (AFP) and carcinoembryonic antigen (CEA) had introduced terminology into which the Regan isoenzyme could not fit. Terms such as oncofetal antigens did not provide for an oncotrophoblast antigen. All of such proteins, however, are products of development which also are reexpressed in neoplasia—hence oncodevelopmental proteins.
In Table I are listed the known tumor isoenzymes of alkaline phosphatase and their developmental counterparts.

L-Leucine-Sensitive Phenotypes of Placental and Tumor Alkaline Phosphatases

If the multiallelic placental genes are reexpressed in cancer cells, would one expect to find them expressed in the same distribution that occurs in normal placenta? A priori one would say "yes." The facts do not support this prediction.

For example, the "D variant" allele (slow-moving on starch gel electrophoresis) appears in less than 1% of normal placentas. We found it to share a biochemical phenotype with the Nagao isoenzyme—both are inhibited by L-leucine. Yet in ovarian cancer, L-leucine-sensitive placental alkaline phosphatase is present in over 50% of the cases [20].

Nagao isoenzyme and D-variant are not identical in their behavior to three peptide inhibitors; L-leucinamide, L-leu-L-leu-L-leu and L-leu-Gly-Gly [4] and in their electrophoretic behavior [21].

Inglis et al [20] observed that the Nagao isoenzyme (placental-type alkaline phosphatase, sensitive to L-leucine) traveled more slowly than the common placental F, FS, and S phenotypes on starch gel electrophoresis. This behavior on starch gel corresponded most closely to the rare D-variant phenotype. The distinguishing property of the Nagao isoenzyme is its specific inhibition by L-leucine, a property not shared by the common F, FS, and S phenotypes. This laboratory then demonstrated that the D-variant phenotype was specifically inhibited by L-leucine.

Doellgast and Fishman [22] found nine leucine sensitive D-variant placentas out of a total 2,323 placentas screened. The D-variant is expressed as a heterozygote with one of the common phenotypes which are not inhibited by L-leucine, the percentage inhibition being 40—50%.

The electrophoretic behavior of L-leucine-sensitive placental alkaline phosphatases have been described by Doellgast and Guenther [23] and two patterns have been described, one in which there is a slow and fast triplet band and one in which the two bands are fused into one. Tumor alkaline phosphatases of this type exhibit a single band.

Non-Regan Isoenzymes—Early Placental-Type Chorionic

Shortly after Regan isoenzyme (term placental alkaline phosphatase) was reported, Timperly [24] found patients' lung cancer tissue rich in an alkaline phosphatase that was different from Regan. It was heat-sensitive and was inhibited by L-homoarginine but not L-phenylalanine, i.e., non-Regan isoenzyme. Subsequently, Sasaki and Fishman [25] observed this non-Regan alkaline phosphatase in ovarian cancer cells where it was demonstrated cytochemically. Mixed populations of ovarian cancer cells expressing both Regan and non-Regan isoenzymes were demonstrated.

The opportunity to study single populations of cells came with the cloning by Singer and Fishman [26] of two HeLa cell lines; one, TCRC-1, was monophenotypic for non-Regan isoenzyme.
TABLE 1. Terminology of Isoenzymes of Alkaline Phosphatases

<table>
<thead>
<tr>
<th>Tumor isoenzymes</th>
<th>Term placental</th>
<th>F, FS, S phenotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regan</td>
<td>Term placental</td>
<td></td>
</tr>
<tr>
<td>Non-Regan</td>
<td>Early placental</td>
<td>(chorionic)</td>
</tr>
<tr>
<td>Nagao [16]</td>
<td>Term placental</td>
<td>D-variant phenotype</td>
</tr>
<tr>
<td>Kasahara [17]</td>
<td>Fast band</td>
<td>Intestine/amnion</td>
</tr>
<tr>
<td></td>
<td>Slow band</td>
<td>Term placental</td>
</tr>
</tbody>
</table>

The question here is, “What is the developmental identity of the non-Regan alkaline phosphatase expressed in cancer cells? The answer was obtained in 1976 by L. Fishman et al [27]. She found that 6-10-week-old human placenta had a species of alkaline phosphatase that matched non-Regan isoenzyme in heat-lability, in its inhibition by L-homoarginine, and in sharing antigenic determinants. The early expression of placental isoenzyme was superseded by the dominance of the well-known term placental alkaline phosphatase in the same sites beginning at 10-12 weeks.

Most interesting in this study was the recognition on microzone cellulose acetate electrophoresis of a fast band A which did not share antigenic determinants with liver/bone, intestinal, or placental isoenzymes.

Such an enzyme band has been detected by Fishman and Singer [28] and Fishman et al [29] in human testes homogenate and in testicular teratocarcinoma. The presence in testis of the term placental type of alkaline phosphatase with L-leucine sensitivity was discovered by Fishman et al [29] and Chang et al [30].

These results have raised the possibility that expression of alkaline phosphatases in tumors and in trophoblast may be attributed to gonadal genes.

Oncodevelopmental Gene Products in Ovarian Cancer

Our interest in ovarian cancer stems from the favorable circumstances it offers the investigator to study tumor proteins. In late stages of the disease, abdominal ascites fluid accumulates owing to the peritoneal spread of implants of tumor cell aggregates. Periodically, this ascitic fluid is collected to relieve the abdominal pressure in the patient. The fluid specimen is frequently rich in tumor cells and tumor cell products. From a set of individual specimens one can determine whether a particular component suspected of being an oncodevelopmental gene product is present or not. From the Fishman laboratory in Boston, those products that were positively identified in ovarian cancer were Regan isoenzyme (term placental alkaline phosphatase) [31,32], non-Regan alkaline phosphatase [25,32], fetal haptoglobin [33], acidic isoferritins [34], histaminase [35], human chorionic gonadotropin [36,32], and CEA [36].

In a study of correlation of Regan isoenzyme and of HCG levels in ascitic fluid, 59% showed Regan isoenzyme and 68% showed HCG, there being a concordance of expression in 55%. Almost invariably the ascitic fluid was
richer in the two placental proteins than the serum when both were collected on the same day. Progressively increasing levels of each placental protein generally correlated with the spread of disease, though there were instances when only one was expressed.

It has been of interest to measure Regan isoenzyme, HCG, CEA and histaminase in the serum and effusion fluids of patients with carcinoma of the ovary, breast, and lung [37]. Levels of serum Regan isoenzyme and HCG did not correlate with the extent of disease in breast cancer patients, whereas CEA did appear to correlate. At least one serum tumor marker was present in 82% of patients with cancer of the ovary. The usefulness of such findings remain a subject of current interest in many oncology centers.

**Gene Expression in Cultured Cells**

The earlier studies on human cancer tissues that brought into view the various developmental alkaline phosphatases also included the presence of Regan isoenzyme in HeLa cells. The latter observation suggested that it should be possible to study oncotrophoblast gene expression under controlled conditions, a circumstance unattainable in the tumor tissues obtained at surgery or at autopsy.

**TCRC-1 and TCRC-2 sublines of HeLa.** The point of departure for this continuing study has been the establishment by Singer and Fishman [26] of two HeLa sublines, TCRC-1 producing Regan isoenzyme and TCRC-2 expressing non-Regan isoenzyme. These two monophenotypic cell lines behave differently in the presence of the corticosteroid hormone, prednisolone; TCRC-1 was relatively rich in β-glucuronidase and in isoferitins [37]. Contemporaneous collaborative studies with Rustigian et al [38] had identified another high Regan alkaline phosphatase producer in the form of a human heteroploid epithelial-like cell line (C-SPT).

The experience of prednisolone enhancement of Regan isoenzyme in TCRC-1 cells in 1974 [26] has itself provided a point of departure for investigating “modulation” of alkaline phosphatase expression and expanding such studies to other cell lines.

**“Modulation” by prednisolone.** Prednisolone regulates the levels of alkaline phosphatase by the enhancement or suppression of specific isozymic forms.

In the cell lines studied, hormone enhancement appears to be specific for the Regan isozyme. Cell lines that possess the Regan isozyme were shown to have elevated activity at its isozyme band position (HeLa TCRC-1 and HEp-2). In the FL amnion cell line, however, the hormone caused the appearance of a Regan band, which was not present in the control. It seems that in some cell lines hormonal enhancement of the initial phenotype occurs while in another cell line (FL amnion) hormone induction of a cryptic phenotype takes place. An additional effect of hormone treatment of the FL amnion and the HEp-2 cell lines was the considerable decrease in enzyme activity of certain other bands. The bands affected in this manner were shown to be intestinal-type alkaline phosphatase. Hormonal regulation in these cell lines clearly appears to be a specific process of altering the relative activity of various isozymic forms produced by the cell population, i.e., “modulation.” The production of the
observed isozyme patterns may be the result of the gene expression of an individual cell or may reflect the contribution of different cell types, each producing a separate isozyme.

If the isozyme pattern is due to different cell populations, each producing its own characteristic enzyme form, then the levels at which the hormone may act are numerous. Hormone treatment could have a differential effect on the proliferation rate of a Regan isozyme-producing population versus an intestinal isozyme-producing one. Another possibility is that this hormone may have a direct effect on the activation of genes that code for each isozyme.

Finally, two interesting observations deserve attention. First, the lack of an increase in specific activity in cells growing in the presence of hormone may obscure alterations in phenotypic expression unless isozyme characterization is carried out. Second, we must now consider the intestinal isozyme as an ectopic carcinoamnion phenotype in the HEp-2 cancer cell line.

This last observation has assumed importance since it has since been found by Japanese workers to correspond to the fast band of the Kashara isoenzyme—found most frequently in human hepatoma.

**Hyperosmolarity.** Corticosteroid hormones are not exclusively endowed with the property of inducing HeLa cell Regan alkaline phosphatase. Simply by increasing the osmolarity of the medium with NaCl, a similar induction occurs, as reported by Fishman and Singer [39]. When cells were grown in hyperosmolar medium plus prednisolone, the resulting increase in specific activity was higher than for the individual agents alone. In both FL amnion and HEp-2 cell lines, the two isozyme bands showed reciprocal behavior with hyperosmolarity as with prednisolone.

The “modulation” could be given a number of interpretations. One possibility is that intestinal-type and Regan isoenzymes may bear a precursor-product relationship to each other in that induction may represent the incorporation of intestinal-type alkaline phosphatase either before or during induction.

The results can also be explained on the basis that prednisolone may stimulate in FL amnion and HEp-2 lines the growth of one population of Regan-producing cells to the extent that they overgrow the intestinal-type cell population. In this connection, the two-cell population explanation is not convincing because the effect of induction is seen in less than 24 hours, which would appear to be insufficient time for the overgrowth to occur.

A third interpretation relates to a gene switch mechanism in which the “turning on” of the Regan gene is accompanied by the “turning off” of the intestinal gene. At this time, there is no evidence for or against this interpretation but it appears to be the most attractive one for further study.

Finally, there may be an influence on the cell cycle in that cells may become redistributed with respect to the periods of the cell cycle and more of them may accumulate in a compartment of elevated gene activity of one phenotype with a subsequent diminution of expression in the other phenotype by virtue of the fewer number of cells in the other compartment.

**Cell cycle.** The events of the cell cycle have been explored by Singer and Fishman [39] in relation to Regan alkaline phosphatase induction. We have found that DNA synthesis is not required for hormone induction as
hydroxyurea, a specific inhibitor of DNA synthesis, did not prevent it. Additionally, when partially synchronized cells were allowed to leave the S period prior to hormone treatment, and hydroxyurea was added to prevent cells from entering the next S period, hormone induction of the Regan isoenzyme was still observed. This indicates that initiation of expression of hormone-induced carcinoplacental alkaline phosphatase occurs prior to the DNA synthetic phase of the cell cycle, probably 8–10 hours before.

**Non-Concordance of Induction by Prednisolone and by Butyrate**

In a paper by Kottel and Fishman [40], five HeLa cell lines have been examined in order to determine the effect of hormone treatment on alkaline phosphatase isoenzyme expression. Both biochemical and immunoelectrophoretic analysis shows that three cell lines demonstrating activity of the placental-type (Regan) isoenzyme in absence of prednisolone are induced to much higher levels after growth in hormone-containing medium for 72 hours. Prednisolone treatment resulted in increased enzyme activity but no change in the isoenzyme profile as determined by microzone and polyacrylamide disc gel electrophoresis. However, the majority of the activity in HeLa line D98AH2 in the absence of hormone was found to be heat-sensitive and inhibited by L-phenylalanine. Immunoelectrophoretic analysis indicated that amnion/intestinal isoenzyme was expressed. No appreciable change in total enzyme activity occurred in the presence of hormone despite the induction of the Regan isoenzyme and the diminution of amnion/intestinal type. Thus treatment of this cell line with hormone resulted in differential expression of the amnion/intestinal and placental isoenzyme components. These results support a regulatory function of prednisolone with respect to expression of alkaline phosphatase isoenzymes in tumor cell lines and identify a HeLa cell line, D98AH2, which elaborates the amnion/intestinal isoenzyme.

This monophenotypic cell line (D98AH2) now can be added to other monophenotypic lines as TCRC-1 or its equivalent HeLa 71, which expresses Regan isoenzyme and TCRC-2 which produces only non-Regan isoenzyme.

Butyrate was first recognized as an inducer of placental proteins in HeLa cells by Ghosh. We have accordingly studied its effects on alkaline phosphatase levels in a variety of cell lines. In four out of five HeLa cell lines, butyrate behaved like prednisolone in inducing Regan alkaline phosphatase. Most interesting was the behavior of D98AH2, which did not respond to butyrate but did respond to prednisolone with respect to Regan alkaline phosphatase. This cell line now offers a valuable opportunity to study modulation by prednisolone as a phenomenon distinct from the modulation by butyrate.

**Biological Uniqueness of Regan Isoenzyme**

Three separate gene loci are known for alkaline phosphatase that code separately for placental, intestinal, and tissues other than placenta or intestine. The placental locus is unique in that it is particularly vulnerable to point mutation and that in cancer there is a disproportionate expression of isozymes that appear closely related to rarely expressed normal alleles. Moreover, such
gene products from tumors rarely are identical in their electrophoretic properties to the common placental isoenzyme phenotypes. Could point mutation rates be greater in tumor tissues?

An observation of potentially great significance was made in 1979 by Doellgast and Benirschke [41] and Goldstein and Harris [42]. They observed that the placentas of the chimpanzee and orangutan monkeys expressed human term placental alkaline phosphatases but that other primate and nonprimate species did not. Both have agreed that the human placental gene constitutes a late evolutionary event. Most recently, Goldstein et al [43] have speculated that this event may have occurred prior to the divergence to the Pongidae (greater apes) and subsequent to the Hylobatidae (lesser apes).

Three important questions have arisen: 1) Does the introduction of late evolutionary genes into the genome render them particularly vulnerable to point mutation? 2) Does the susceptibility of one gene to point mutation subject its neighboring genes to alterations in gene arrangement and gene expression? 3) Why is the placental alkaline phosphatase gene especially susceptible to allelic expression in both trophoblast and tumor?

**Regan Isoenzyme Tumor Marker?**

The clinical evidence so far would indicate that the expression of Regan isoenzyme is seen most frequently in patients with gonadal and urologic malignancies. The frequency is in the order of one out of two patients. In a number of instances in the literature, the Regan isoenzyme level in ascites fluid and in serum parallels the clinical course but in other cases it does not. It is also true that low levels of this gene product register in the circulation in presumably normal subjects and that disease conditions often but not always associated with a predisposition to cancer may show elevations in serum Regan isoenzyme. It is certainly not a general tumor marker.

Tumor markers become useful in the differential diagnosis of cancer in a population of high rather than of low prevalence, e.g., prostatic acid phosphatase in patients coming to a urologist’s office or clinic or calcitonin levels in a kindred with high prevalence of medullary thyroid carcinoma.

Recent studies point to a positive correlation of Regan isoenzyme with seminoma. Thus high serum levels of Regan isoenzyme and gamma-glutamyl transferase (GGT) were found in patients with seminoma [44]. In addition, evidence was obtained for the presence of the early placental-like isoenzyme in serum which had unusual heat stability and electrophoretic behavior. Also, Swedish workers [45] have found that six out of seven seminoma patients exhibit Regan isoenzyme in their serum and that both AFP and Regan isoenzyme levels followed the clinical course well. Uchida et al in their unpublished work demonstrated Regan isoenzyme by immunoperoxidase technique in paraffin sections of 10 out of 11 seminomas. They have proposed Regan isoenzyme as a marker for seminoma.

There does seem to be a reasonable rationale for the seminoma-Regan isoenzyme correlation. Since testis and ovary normally do produce placental-type alkaline phosphatase, the overproduction of the enzyme in
gonadal tumors could be interpreted as due to the multiplication or enhanced expression of active gonadal genes, an example of so-called eutopic gene expression. However, expression of Regan isoenzyme in lung cancer is an example of ectopic gene expression. Question? Is the Regan isoenzyme a trophoblast or gonadal gene product? This is a fundamental question which has come from the clinical experience and needs resolution.

Oncodevelopmental Proteins as Tumor Markers

Certainly AFP has proved useful in the diagnosis of yolk sac tumor, as preembryonic yolk sac is a tremendous producer of AFP. Also, HCG is a marker of trophoblast cells which populate certain testicular tumors and HCG is a gene product of the early placenta and of testis.

The recognition of either one or both of these markers in tissue and/or serum gives the clinician confirming evidence of where to look for the tumor being diagnosed and is of definite utility in the management of patients with tumors of yolk sac or of trophoblast elements.

Often one encounters a marker that was seen in the original tumor which disappears during the course of the disease. In this case, the “marker” gets a black eye in the opinion of some. However, the disappearance of the marker is telling us something about neoplastic differentiation because surely these cells are adapting themselves to their perpetuation in the patient. Is there a marker for this new stage in development of the neoplastic phenotype? The fact that none of these are in our possession is the strongest reason to intensify research in oncodevelopmental biology to the end of discovering these later markers.

Finally, it should be stated that a gene product that makes its appearance at the expected time and place in embryonic development is a “differentiation” marker, whereas the same gene product expressed in the wrong developmental time and place becomes a “tumor” marker. Clearly, discoveries in developmental biology have relevance in oncology and vice versa.

REFERENCES


