# Characterization of Retinoblastoma Gene Expression in Canine Tissue and Canine Osteosarcoma

Ann Underwood

University Undergraduate Fellow, 1990 - 1991

Texas A & M University

Department of Veterinary Physiology and Pharmacology

**APPROVED:**

Fellows Advisor Deborah J. Kocheran, D.V.M., Ph.D.

Honors Program Director

## TABLE OF CONTENTS

## LIST OF FIGURES AND TABLES:

## PROJECT SUMMARY

The retinoblastoma gene is the prototype for a class of recessive cancer genes in which loss of activity of both normal alleles is associated with the occurrence of cancer. Deletions occurring at the RB1 locus on chromosome 13q14 have been implicated in the formation of retinoblastoma. Deletions of the RB1 locus have also been noted in several cases of osteosarcoma. Mutated genes transcribe a mRNA 4.0kb in length as compared to the normal length of 4.7kb.

RNA has been analyzed from canine tissues and OS tumors. Characterizing the mRNA of the RB gene in canine tissues and tumors will allow evaluation of canine osteosarcoma as a molecular model for the human disease and may enable dogs to be used as research models for the benefit of both species.

Total RNA was extracted from samples using guanidinium thiocyanate and phenol/chloroform. After quantification, RNA was electrophoresed through horizontal agarose gels. Gels were stained with ethidium bromide and examined for intact RNA. Nucleic acids were transferred overnight to a Nytran membrane. The membranes were hybridized with a $^{32}$P-labelled human Rb cDNA and the size of the Rb message analyzed.

It was anticipated that a decreased transcript size in some osteosarcoma tissue would indicate that a deletion of the RB gene had occurred. Hybridizations of the Rb probe to RNA from normal tissues revealed a typical transcript of approximately 6kb. Attempts to optimize hybridizations between the Rb cDNA and canine osteosarcoma RNA have been unsuccessful but are continuing.

## BACKGROUND AND SIGNIFICANCE

### Oncogenes and Anti-Oncogenes

Cancer cells exhibit many abnormal traits. The most obvious characteristic is loss of contact inhibition. Cancer cells continue dividing even when crowded; they stack on top of each other to form an aggregated mass, eventually forming a tumor. They have a different shape than non-cancerous cells; they rely on a greater extent on anaerobic metabolism. The outer membrane displays special tumor antigens, which confer distinct immunological properties on the cell (26). Cancer cells, unlike normal cells, will grow in semi-solid media and will form tumors when implanted into nude mice.

A relatively small number of genes, termed oncogenes or proto-oncogenes, may profoundly influence transformation of a cellular phenotype from normal to neoplastic (26). Oncogenes are converted from normal cellular genes, the proto-oncogenes. Proto-oncogenes may be converted to active oncogenes by a number of somatic and/or hereditary events. Oncogene mutations as discrete as a single base pair change resulting in a single amino acid substitution have been shown, in some cases, to promote a transformed phenotype. Various oncoproteins may promote cellular growth, regulate cellular signaling and/or regulate transcriptional rates (18). The mechanisms by which oncoproteins work are diverse and clearly involve fundamental cellular processes and changes in regulation of these processes.

More recently, a new mechanism for the development of cancer has been studied. In this mechanism, certain proteins act in normal cells not to promote proliferation but to suppress it (27). Thus, it is the loss of "growth-suppressor" proteins that causes unregulated cell growth by removing a normally present control. Genes encoding such growth-suppressor proteins have been termed anti-oncogenes, or recessive oncogenes. "The essential difference between a dominant and a recessive oncogene is that for the former it is the presence of the product, whereas for the latter it

is the absence of the product, that leads to transformation." (13).

Retinoblastoma and Osteosarcoma

Retinoblastoma serves as a prototype for this recessive class of cancer genes. The normal function of the RB gene is to suppress growth (27). Loss of function is thus associated with the appearance of malignancy. Specific changes resulting in homozygosity or hemizygosity for the mutant or inactive allele appear to be a key mechanism leading to tumor formation (25).

Retinoblastoma is a rare cancer found in the retinoblasts of children. Retinoblasts are the precursors of retinal cells. After differentiation into a specialized retinal cell, a retinoblast stops dividing and no longer serves as a target for tumorigenesis. Retinoblastoma occurs with a frequency of 1 in 10,000 (2) to 1 in 20,000 live births (24), where the age of onset is anywhere from 0 to 7 years. No discernable geographic, racial or sex-specific clustering was apparent in the cases studied (7). Affected individuals fall into two basic categories: a) those having multifocal tumors that develop at an earlier age and b) those with unilateral tumors that appear at a later age. Individuals affected with multifocal tumors tend to be affected with the hereditary form of retinoblastoma, while those with unilateral tumors are usually affected with the sporadic form.

In 1971, Knudson hypothesized that retinoblastoma results from a mutation in both alleles of chromosome 13 (19). Two events are necessary in the development of both the hereditary and the sporadic forms or retinoblastoma. The first event is a germinal mutation in the hereditary form and a somatic mutation in the sporadic form. The second event is somatic in both forms (20). In hereditary cases, the mutant allele is usually inherited from the father (3) and is present in all cells of the body. As a result, only one additional mutation must occur in the retinoblast in order for cancer to develop. With the sporadic form, both mutations must occur somatically.

Studies have shown that the tumor predisposing locus, RB1, is within arm q14 of chromosome 13. This allele can be inherited in a Mendelian fashion and is responsible for familial

and sporadic tumors (10). Tumors arise in cases having a loss of constitutional heterozygosity for chromosome 13 (7), resulting in loss of the tumor suppressing phenotype (16).

The heritable form of Rb is transmitted as an autosomal dominant cancer susceptibility trait (22). Each of the offspring of the carrier parent has a 50% chance of inheriting the trait, and, of these, 90% will develop Rb (22). While the predisposing mutation is inherited in a dominant fashion, it is recessive at the cellular level. The gene represents the prototype for a recessive class of human tumor cancer genes (27). The inherited mutation is not sufficient to cause cancer. It merely predisposes the cell to the development of retinoblastoma. For cancer to develop, the second allele must also undergo mutation.

The method by which the second mutation occurs is not known. Possibilities include nondisjunction, nondisjunction with reduplication of the mutated allele and mitotic recombination. Any of these events results in homozygosity or hemizygosity for the abnormal chromosome 13. (Figure 1) The mutation apparently can be caused by either a microscopic or submicroscopic change (20). Whether or not these tumors have identifiable structural changes within the Rb gene, they either have an absence or abnormal expression of the Rb transcript (2). Normal retinoblasts produce a Rb mRNA of 4.7kb. In the retinoblastoma cell line Y79, a Rb mRNA of only 4.0kb is expressed (22). (Figure 2) Through reverse transcription, it has been shown that 470 nucleotides (276-745) are lost near the 5' end of the gene (22). This sequence correlates with removal of exons 2-6, which results in frame shift and premature termination of translation in exon 7 (22). As the size of the deletion is only about 50kb , it is often too small to be cytogenetically visible (22).

The normal protein produced by the Rb gene is 110 kD in size (22). Rb tumors lack this protein. Experiments have shown that in normal cells, the protein is located within the nucleus, where it is associated with DNA binding activity (22). This association supports the proposed role of the protein in regulating other genes. It is these genes which, when unregulated, cause the

uncontrolled proliferation seen in cancer cells.

It has been noted that individuals affected with the hereditary form of retinoblastoma show a greatly increased risk of developing independent secondary neoplasms. The most common of these secondary neoplasms is osteosarcoma (OS). Studies show that cells in some osteosarcoma tumors exhibit a deletion at the Rb1 locus. It is therefore thought that some OS arises by the same mechanism as retinoblastoma (7). Other etiologic possibilities have been suggested for OS. OS caused primarily by radiation effects has been described, but studies have also revealed cases in which OS developed outside the field of radiation or in the absence of such treatment (15). (Figure 3)

Comparative Features of Canine Osteosarcoma

Osteosarcoma in dogs is the most commonly occurring skeletal neoplasm. It affects 7 in 100,000 dogs annually. Dogs diagnosed with OS have a very poor prognosis, with only 10-20% surviving one year following amputation. As in humans, OS readily metastasizes, with the most common site being the lungs.

Many other similarities exist between OS in man and dog. (Table 1) OS primarily affects the appendicular skeleton of both species. The bones most often affected are the proximal humerus, the distal femur, the proximal tibia, and the distal radius. A genetic correlation is seen, with large breeds of dogs (over 80 lbs.) and tall humans being more commonly affected. Familial trends are also seen. There is a high occurrence of OS among families of St. Bernards and Rottweilers and in several pairs of siblings in man (14).

The tumors also share the same histologic appearance and some antigenic properties. Histologically, there are three major subtypes of osteosarcoma, depending on the primary cell component: osteoblastic, fibroblastic, and chondroplastic. Monoclonal antibodies TP-1 and TP-3 have been shown to stain all OS cells in humans and dogs while being excluded by normal cells.

This fact will allow the use of monoclonal antibodies in OS detection and treatment in dogs and man (14).

Treatment

Current treatment for human osteosarcoma involves removal of the tumor followed by radiation therapy and chemotherapy. In vitro research is currently underway to test the possibility of gene therapy as a mode of treatment. Huang et.al. demonstrated that by inserting a functional RB protein into cells containing inactivated endogenous RB genes, the neoplastic phenotype was suppressed. Cells were infected with a retrovirus carrying the functional RB gene. Infected cells in culture subsequently expressed a RB protein indistinguishable from the native RB in terms of molecular weight, cellular location and phosphorylation (17). Infected cells also became enlarged and exhibited severely inhibited growth (17). When injected into nude mice, the infected cells lacked the ability to form tumors.

Furthermore, Bookstein et.al. successfully restored normal RB expression in human prostate cancer cell line DU145. DU145 was shown to possess a RB mRNA transcript that lacked 105 nucleotides encoded by exon 21 (3). Normal RB expression was restored in DU145 cells using retrovirus mediated gene transfer. The transformed cells, expressing exogenous RB protein, subsequently lost their ability to form tumors in nude mice (3).

The results obtained by Huang and Bookstein illustrate the possibility of gene therapy as a new form of treatment of malignant cancers. Gene therapy would be beneficial in that it is based on the permanent correction of an underlying defect in tumor cells. Unlike conventional cytotoxic cancer therapies, gene therapy should not be harmful to normal cells and therefore need not be specifically targeted (17).

It is hoped that by studying OS in canines, a correlation can be found between canines and humans. If OS in dogs is shown to share molecular features with human Rb and OS, dogs with hereditary diseases may then be considered as research models for the benefit of both species.

## SPECIFIC AIMS

1. Using Northern analysis, to characterize RB gene transcription in normal canine tissues.
2. Using Northern analysis, to characterize RB gene transcription in canine osteosarcoma.
3. To determine if the Rb transcript from canine osteosarcomas differs from the transcript of normal tissues. To determine the frequency of Rb mRNA changes in canine osteosarcoma.

## MATERIALS AND METHODS

Tissue Samples and Cell Lines

Normal tissues (n=6) were collected from terminal teaching dogs immediately after euthanasia. No history was available on these animals.

Canine osteosarcoma tissue was collected from animals presented for tumor biopsy or removal at the Colorado State University Veterinary Teaching Hospital (n=24) and the Texas A&M Veterinary Teaching Hospital (n=6). All tissue and tumor samples were stored at -80$^{o}$ C until use.

Two human cell lines were also utilized. Human retinoblastoma cell line, Y79, is known to express a truncated Rb message of 4.0kb. A human fibroblast cell line, CCD-45, is expected to express a normal Rb transcript of 4.7kb. Both cell lines served as homologous controls for hybridization with the human Rb cDNA probe. Y-79 cells were grown in RPMI with 15% fetal calf serum; CCD-45 cells were grown in MEM with 10% FCS. Both cell lines were grown at 37$^{o}$C in 5% $CO_2$ and 95% $O_2$.

RNA Extractions

Approximately 0.5g of frozen tissue and tumor samples was ground under liquid $N_2$ using a mortar and pestle. Following grinding, the samples were denatured and solubilized in guanidinium thiocyanate solution (4M guanidinium thiocyanate, 2-mercaptoethanol). The tissue

was passed through a 16G needle followed by a 20G needle to reduce viscosity. Sodium acetate was added to lower pH. The samples were extracted with phenol followed by chloroform:isoamyl alcohol (24:1). The aqueous phase was precipitated with equal volume isopropanol at -20°C for 1.5 hrs. The pellet was resuspended in guanidinium solution and reextracted with phenol and chloroform:isoamyl alcohol. The aqueous phase was precipitated again under the same conditions. The pellet was resuspended and precipitated with 2.5 vol. ethanol. The new pellet was washed in 70% ethanol, dried and resuspended in 100mcl 0.5% SDS.

Quantification of RNA

Extracted RNA was diluted 1:20 in 0.5% SDS and quantitated using a Beckman DU-70 spectrophotometer. The spectrophotometer was used on the dual wavelength program and set to compare O.D.260 and O.D. 280. The 260/280 ratio should be close to 2.0 and not less than 1.6 for acceptable RNA purification. Calculations used were as follows:

mcg RNA= (O.D.260) (constant) (dilution factor) (ml. sample)

constant = 40mcg/ml

dil. factor = 20 (5mcl/100mcl=1/20)

ml. sample = 100mcl-5mcl = 0.095

Nucleic Acid Electrophoresis

10mcg of sample were dried and resuspended in 10mcl sample buffer [37% formaldehyde (17.5%vol), 2x di-formamide (50%), 5x MOPS running buffer (10%), TE pH8.0 (22.5%)]. 5mcl of 6x tracking dye was added and the samples were loaded into the wells of a 0.5% agarose horizontal gel. 4mcl ethidium bromide were added to the gel to stain the nucleic acids during electrophoresis. The gel was run in 1x MOPS running buffer at 50V for 3-4 hrs. A UV illuminated photograph was taken of each gel for comparison of rRNA band size. The nucleic acids were then transferred overnight from the gel to a Nytran membrane using 1x sodium transfer

buffer (pH 6.5).

Probe Labelling

3.8R and 0.9R human cDNA probes were prepared; 3.8R hybridizes to the 3'end and 0.9R to the 5' end of the Rb transcript. The probes were labelled using the BRL Random Primers DNA Labeling System. 50ng of DNA was added to 23mcl 1xTE and denatured. 6mcl dNTPs, 15mcl random primer buffer solution, and 5mcl $^{32}$P dCTP were added on ice and mixed. 1mcl Klenow fragment was added and the mixture briefly centrifuged. The probe was allowed to incubate at 37'C for 30 min. Equal volume stop buffer was added and the resulting mixture was separated through a column of Sephadex G50 with TE. The labelled probe was collected. 1mcl was spotted on a glass fiber filter (Whatman GF/C) and counted using a LKB 1211 RackBeta Liquid Scintillation Counter.

Northern Hybridization

Membranes were hybridized using a rapid hybridization protocol. Northern blots were prehybridized with shaking at 65$^{o}$C for 15 min in rapid hybridization buffer (pre-warmed to 65$^{o}$C). The probe was denatured at 95-100$^{o}$C for 2-5 min. The probe was added to the hybridization buffer at a concentration of $1x10^{6}$ cpm/mcl. The membranes were allowed to hybridize with shaking at 65$^{o}$C overnight (minimum of 2 hrs. recommended in protocol). The membranes were washed once at room temperature for 15 min. in 1% SDS and 1xSSC, then twice at 65'C for 30 min. in 0.5% SDS and 0.5xSSC. The washed membranes were placed in a seal-a-meal bag and autoradiographed with 2 intensifying screens at -80$^{o}$C for 48-120 hrs.

Analysis

The developed autoradiographs were analyzed by comparison to known molecular weight markers to determine the size of the Rb transcript in normal tissues and in canine osteosarcomas.

## RESULTS

### RNA Extraction

Total RNA was extracted from tissues normal canine tissues, control cell lines, and canine osteosarcomas. RNA was quantitated and evaluated for intactness (Tables 2 and 3).

Since nucleic acids absorb at a wavelength of 260nm and proteins absorb wavelengths of 280nm, the desired range for the 260/280 ratio is 1.6 to 2.0. Most samples yielded a ratio in the desired range, while some (103573, 105989,106463, 106685, 106899-B, OS2.7) had lower ratios, indicating the presence of excess protein. Only 3 samples did not yield at least 10mcg of RNA (091091, 099580, 107393). The entire quantity of RNA available for these three samples was loaded per lane during electrophoresis.

### Nucleic Acid Electrophoresis

CCD-45 is a normal human fibroblast cell line and as such is expected to express a typical RB mRNA of 4.7kb. Y-79, a cell line derived from human retinoblastoma, expresses a shortened mRNA transcript (4.0kb). RNA from both of these cell lines were included as controls.

The integrity of RNA from all samples was evaluated. Following electrophoresis for 3-4 hours, the gels were examined under UV light for bands in the regions of the 18S and 28S subunits of rRNA, indicating the presence of intact RNA. These bands were seen for all normal tissues (Fig. 4 and 5), 3 lanes of CCD (Fig. 4-9), one lane of Y-79 (Fig. 7), and 14 lanes of tumors (Figs. 6-9). Other lanes indicated the presence of degraded RNA. (See Figs. 4-9 and Tables 2 and 3)

### Northern Hybridization

Nucleic acids were transferred from gels to Nytran membranes. Following transfer, the membrane was probed using 3.8R and 0.9R. At no time were acceptable hybridization signals apparent with the 3.8R probe. Using 0.9R, signals were obtained for CCD45 and 5 normal

tissues (lymph node, liver, testes, kidney, uterus) (Fig. 10). However, no signal was seen with any of the later membranes. Possible signals were obtained on some tumors, but were so faint as to be inconclusive.

## DISCUSSION

### Extraction of Samples

Total cellular RNA was extracted from six normal canine tissues, 30 osteosarcoma tumors, and two human cell lines. The quantity and quality of RNA obtained were calculated and are shown in Tables 2 and 3. The majority of samples yielded high amounts of RNA, with the exception of 091091, 099580, and 107393. Ratios obtained for 260/280 were primarily between 1.6 and 2.0. Ratios less than that indicate the presence of unextracted protein contaminating the DNA.

### Nucleic Acid Electrophoresis

All electrophoresis was accomplished using an RNA ladder for determination of transcript size and RNA from CCD-45 cells for comparison to typical human Rb transcript. RNA from Y-79 cells was included on three of the gels, until the available quantity ran out. Y79 Rb transcripts are known to be truncated and thus represent an important positive control for Rb gene mutation. Following electrophoresis for 3-4 hours, the gels were examined for the presence of intact rRNA. Bands localized at the 18S and 28S subunits of rRNA suggest the presence of undegraded RNA. In some cases where the bands were not seen, clumps of degraded RNA were observed at the end of the lane. Other lanes showed no evidence of nucleic acids (Figs. 4-9).

The rRNA bands were seen for all normal tissues, CCD-45, Y-79, and for 14 out of 30 tumors. There are many possible explanations for the lack of intact RNA. 24 of the tumors were sent to Texas A&M from Colorado State University. It is not known how the tumors were

handled before their arrival. Perhaps they were allowed to sit at room temperature for a period of time before freezing. The manner in which the samples were shipped could also have been less than ideal. If at any time the samples were allowed to thaw, it would allow time for endogenous RNAses to degrade the RNA.

The error may also lie within the extraction procedure. I extracted the tumors in 3 batches of 10. From the first extraction, 5 tumors indicated intact RNA; from the second, only 2 had intact RNA; and from the third, 7. One might therefore assume that mishandling occurred, especially during the second batch. However, mistakes made during the extraction cannot account for the degradation seen in all the samples as some from each batch, as well as all normal tissues and cells, had intact RNA.

Northern Hybridization

The success with which the probe bound to the canine RNA was minimal. All membranes were hybridized first with 3.8R then with 0.9R. It was hoped that the size of the mRNA could be determined by analyzing the location of the bands. However, as none of the mRNA was successfully labelled with the radioactive probe, no bands were found.

When using the 0.9R probe, which binds to the 5' end of the transcript, hybridization was seen with one membrane. Signals were detected with CCD45 and 5 normal tissues (lymph node, liver, testes, kidney and uterus) (Fig. 10). The approximate transcript size of mRNA from normal tissues and CCD45 cells was 2.4 kb. Another band around 6.5kb was found for the CCD45 cells and for LN-4, U-5, and L-2. One tumor (107393) did hybridize to the 0.9R probe. The approximate transcript size was 2.5kb, with another band at 7.5kb (Fig. 11).

I am unsure as to exactly why the hybridizations were unsuccessful. Lack of homology between canine RNA and a human DNA probe was one possibility. However, the human probe should have labelled the RNA from the CCD and Y-79 cells, both of which were derived from

human samples. Also, the 0.9R probe hybridized with normal canine tissues at an earlier date. It would therefore be reasonable to expect a signal from the other normals probed with 0.9R at a later date. In addition, if homology exists between human probe and normal canine tissue RNA, the same homology should exist with canine tumor RNA.

Since lack of homology can essentially be ruled out, other reasons must be explored. A problem may have existed with the probe itself. Perhaps the $^{32}P$ dCTP was not fresh or did not incorporate adequately into the DNA. However, on the basis of the specific activity, it is thought that the probe should have been adequate.

The probe DNA may not have been allowed to denature long enough before being added to the membrane. Repetition of this technical error on all blots would have been unlikely.

Another possible cause lies in the transfer of the nucleic acids from the gel to the Nytran membrane. All transfers were allowed to proceed overnight, and all were set up in the manner detailed in the protocol. When dismantling the transfer, in each case it was noted that the tracking dye had transferred from the gel to the membrane, indicating that the nucleic acids should also have transferred.

Steps that can be taken in the future to help increase hybridization include a) cloning the canine RB gene to create a homologous cDNA probe, b) preparing oligonucleotide probes to conserved areas of human RB gene that might be expected to hybridize more efficiently with canine transcripts and/or c) using a reverse polymerase chain reaction (rPCR) to amplify the canine RB message. These methods increase the possibility of documenting a hybridization for the canine Rb transcript.

Cloning of the canine RB gene would require production of a canine cDNA library from canine mRNA. Screening using nucleic acid probes to RB sequences or antibodies to expressed Rb fusion proteins should allow selection of positive clones with subsequent sub-cloning and RB

cDNA purification. This procedure would evenutally allow for a homologous Northern screening system of canine osteosarcomas using a canine RB cDNA probe. The investment of time and energy for this approach would be substantial.

A more practical method would be to prepare oligonucleotide probes for conserved segments of the human RB gene. Since the sequence of the human Rb gene is known and functional regions of the gene delineated, a specific sequence from a conserved region of the gene can be synthesized. Conserved gene sequences in the human gene would presumably be represented in canines allowing more efficient hybridization.

Another possibility would be to use reverse polymerase chain reaction to amplify canine Rb mRNA. Canine mRNA would be reverse transcribed to DNA which would then be used in the PCR reactions. Two oligonucleotide primers flanking the desired segment of Rb DNA and oriented with their 3' ends facing each other would be designed so that DNA synthesis extended across the DNA segment between them. The template DNA would be denatured in the presence of the oligo primers and the 4 dNTPs. The mixture would be cooled allowing the oligos to anneal to the target segment. Annealed primers would then be extended by DNA polymerase. After repeating the cycle several times (~30x), an amplification level of $10^6$ of the desired segment should have occurred. This procedure would yield a much greater amount of canine Rb mRNA available for Northern hybridizations (23).

## CONCLUSION

While many explanations exist for lack of adequate hybridization with the Rb probe, none provides an adequate explanation in itself. The cause may be a factor of all those reasons detailed or may be something I have not considered. However, while no signal was obtained for the tumors, signal was obtained for some normal tissues. Also, the RNA from the tumors remaining after electrophoresis has been quantitated and stored at -80°C. Thus, it is feasible that given more

time, the errors and drawbacks encountered could be worked out and sufficient data collected from the tumors to allow analysis of Rb transcripts.

With the data collected, the Rb gene can be characterized in the canine species and research into osteosarcoma and possible treatments including gene therapy can continue. Using the data already collected, it has been possible to characterize Rb transcripts as found in normal canine tissues as compared to the human fibroblast cell line CCD-45.

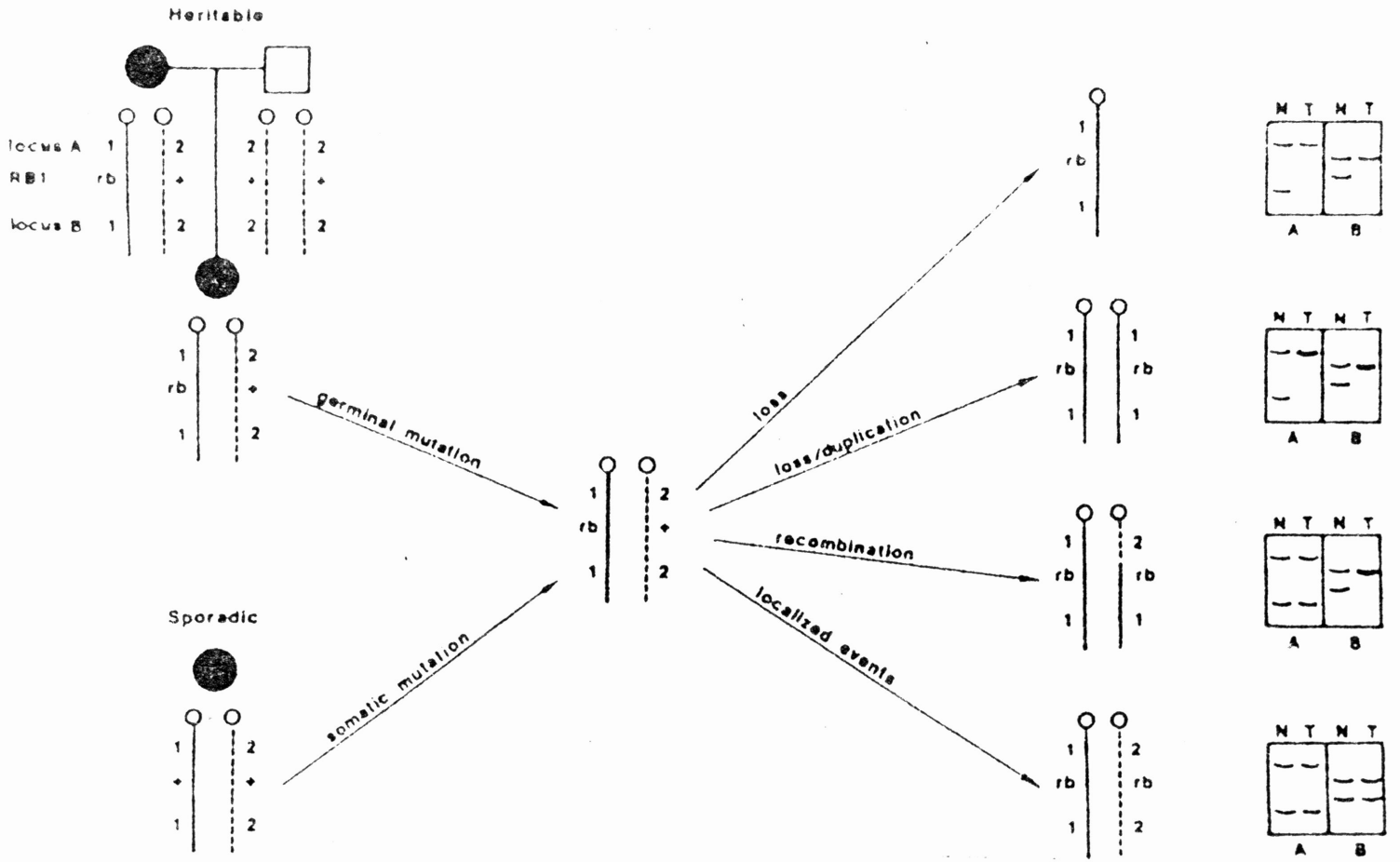


Fig. 1. Proposed mechanisms for the occurrence of retinoblastoma (19, 831).

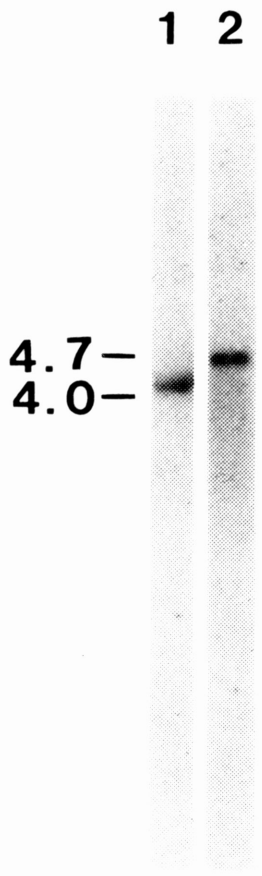


Fig. 2. Comparison of message size seen from mRNA of fetal retina vs. mRNA from retinoblastoma Y79 cells. (14, 6018)

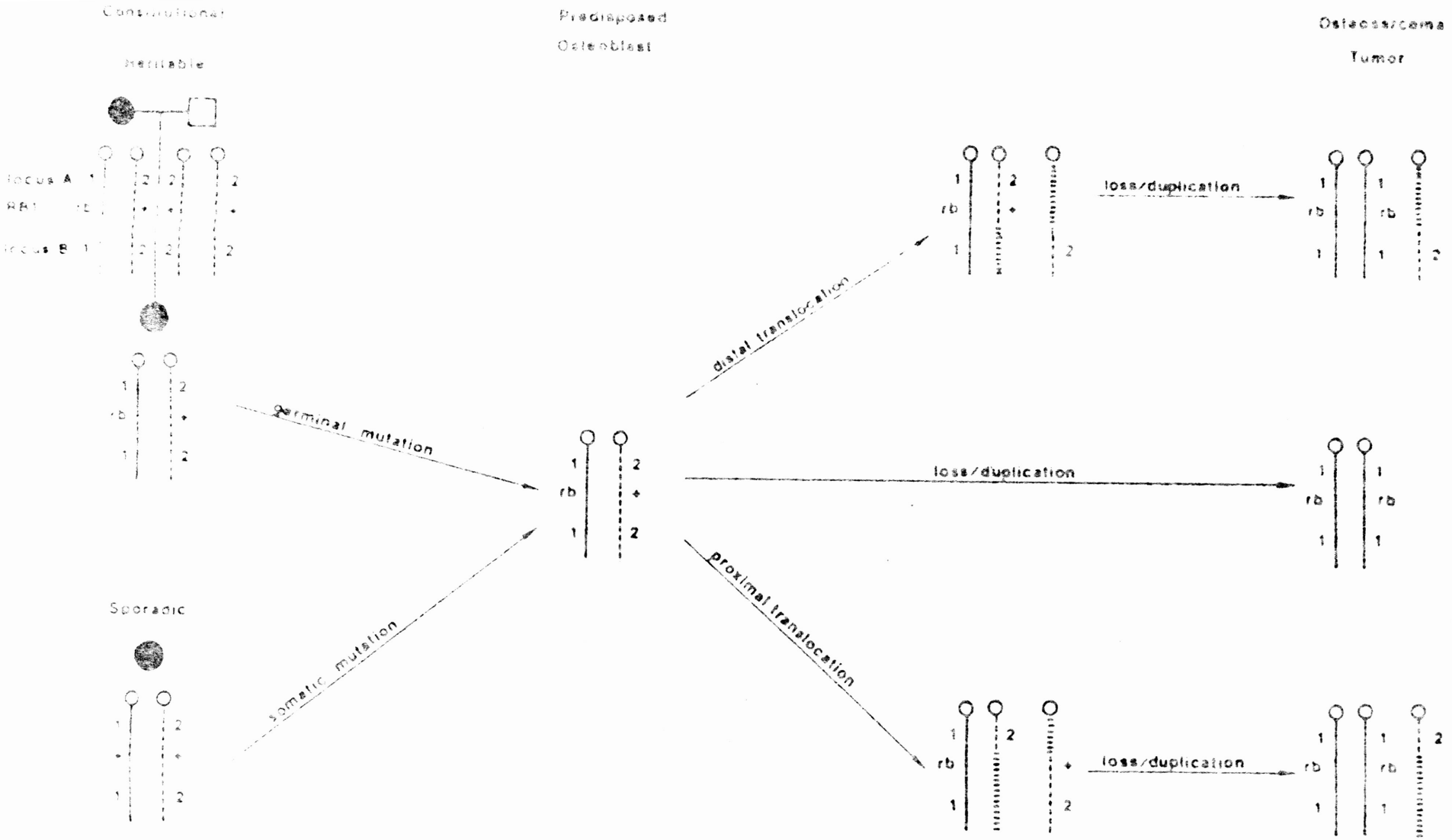


Fig. 3. Proposed mechanisms for the oncogenesis of osteosarcoma.

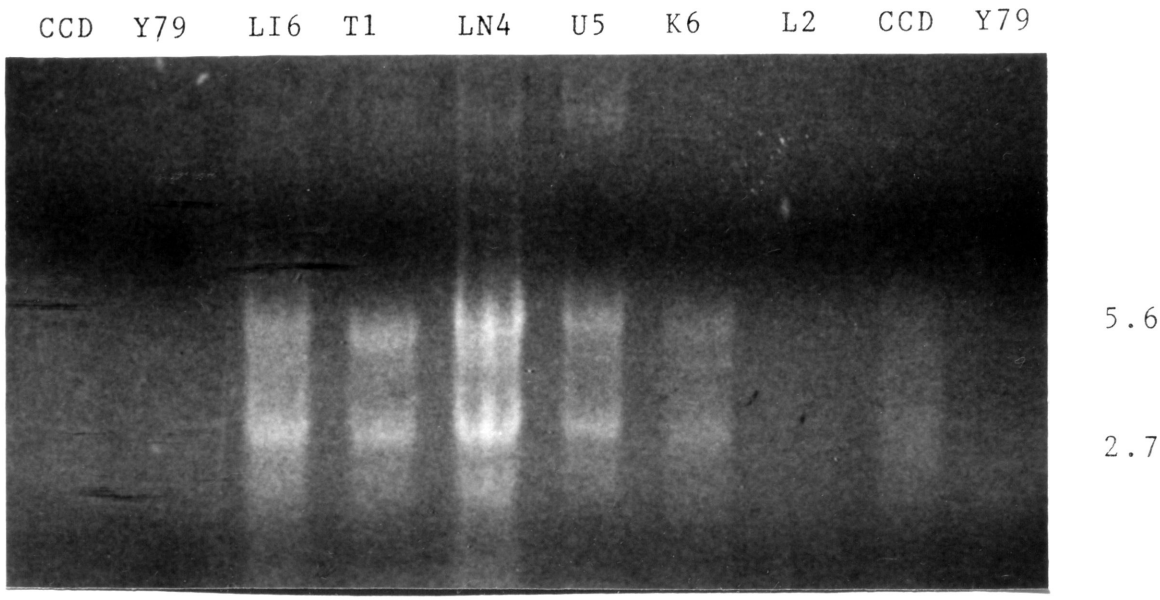


Fig.4. Horizontal agarose gel electrophoresis of RNA from normal tissues. 10mcg RNA were loaded per lane, stained with EtBr, and run for 3-4 hrs. Lane numbers correspond to tissues found in Table 3.

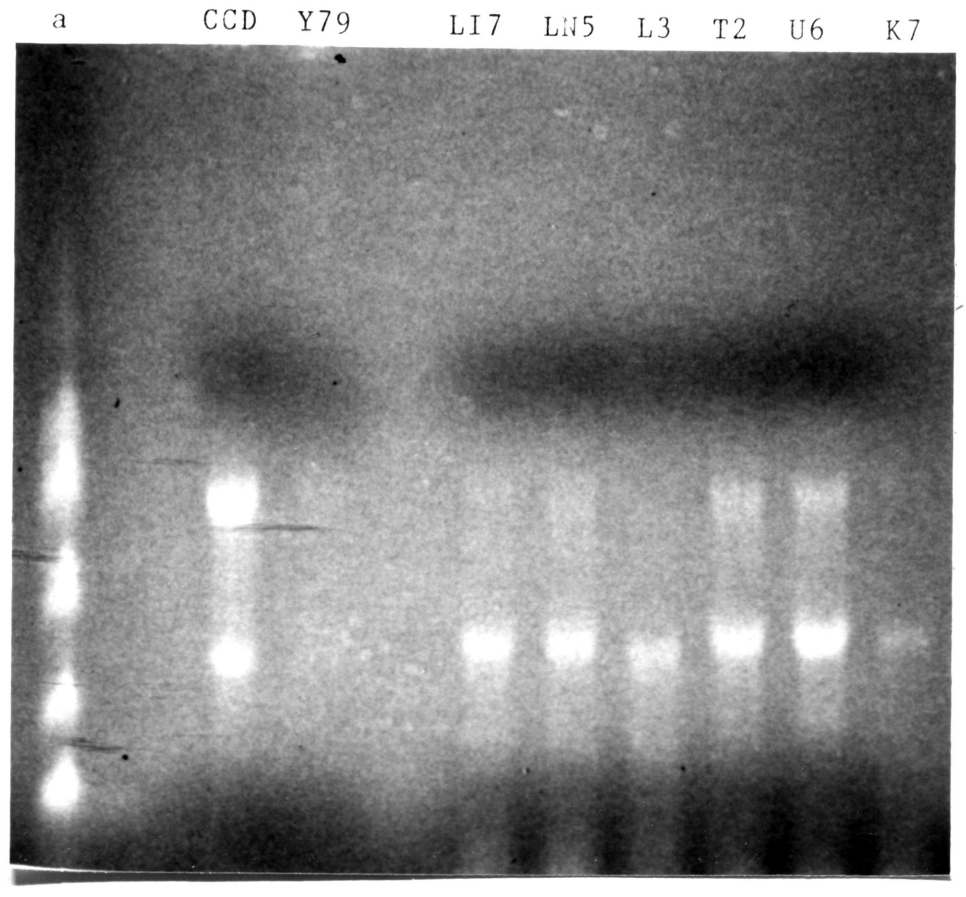


Fig.5. Horizontal agarose gel electrophoresis of RNA from normal tissues. Prepared as in Fig.4. Lane numbers correspond with Table 3. Lane a contains RNA size markers.

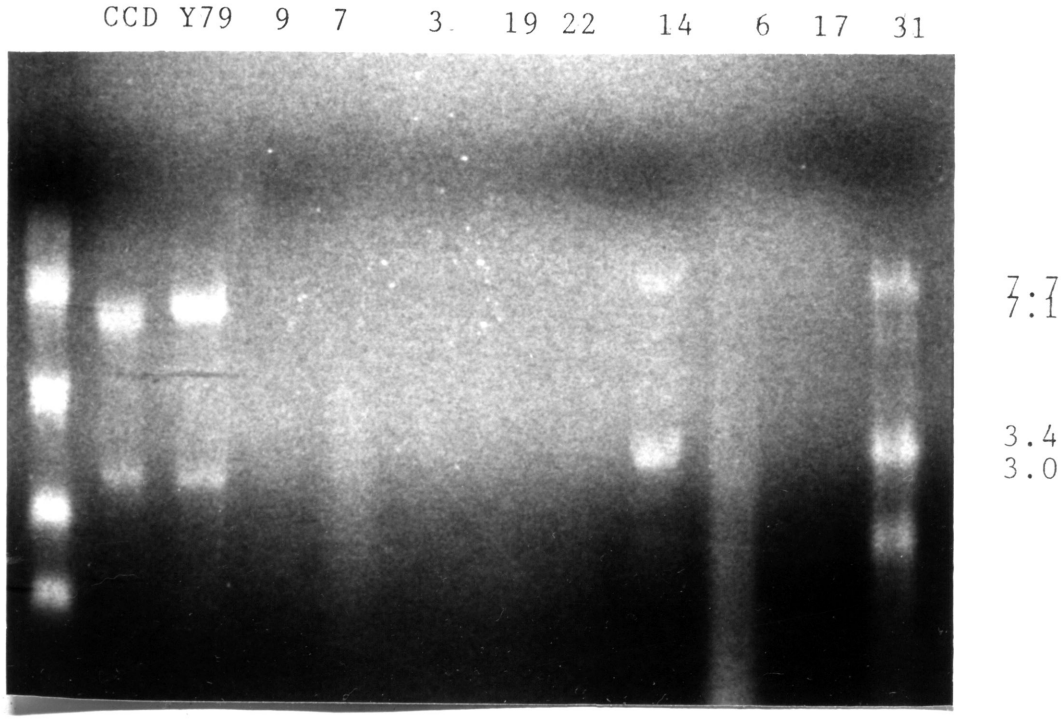


Fig.6. Horizontal agarose gel electrophoresis of RNA from OS tumors. Prepared as detailed in Fig.4. Lane numbers correspond with Table 2.

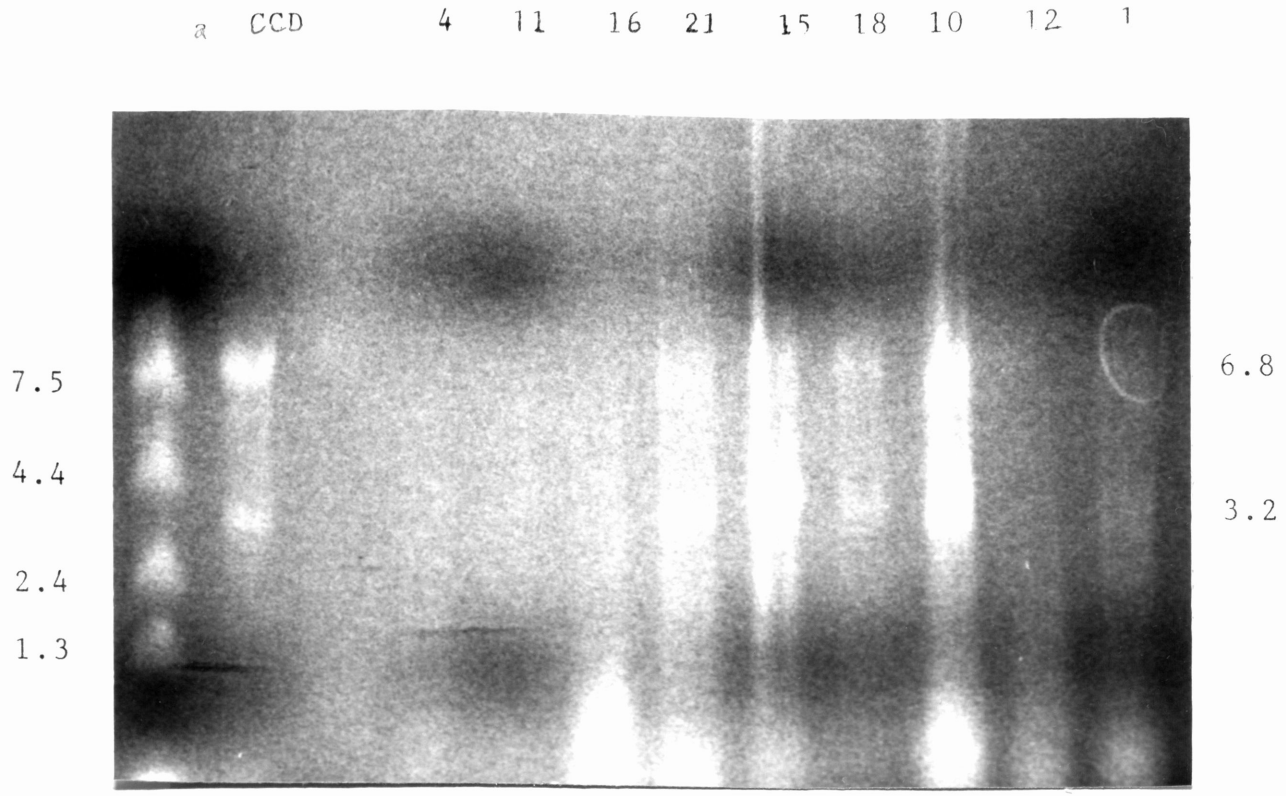


Fig.7. Horizontal agarose gel electrophoresis of RNA from OS tumors. Prepared as detailed in Fig.4. Lane numbers correspond with Table 2. Lane a contains RNA size markers.

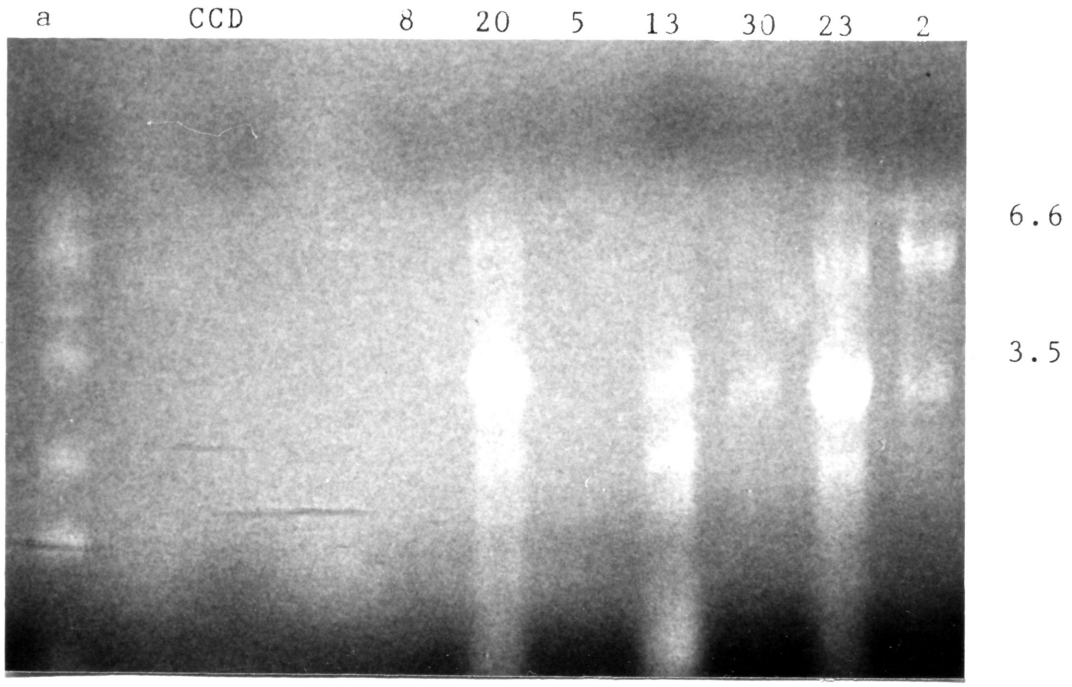


Fig.8. Horizontal agarose gel electrophoresis of RNA from OS tumors. Prepared as in Fig.4. Lane numbers correspond to Table 2. Lane a contains RNA size markers.

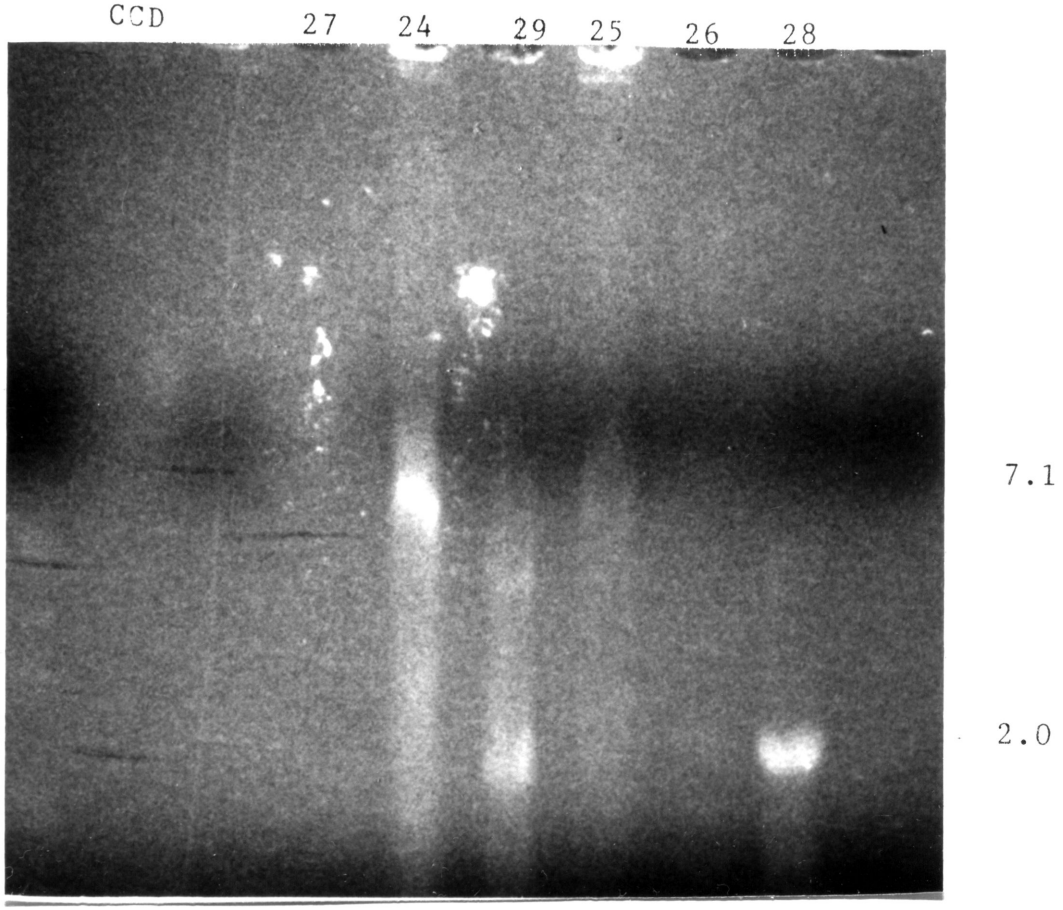


Fig.9. Horizontal agarose gel electrophoresis of RNA from OS tumors. Prepared as in Fig.4. Lane numbers correspond to Table 2.

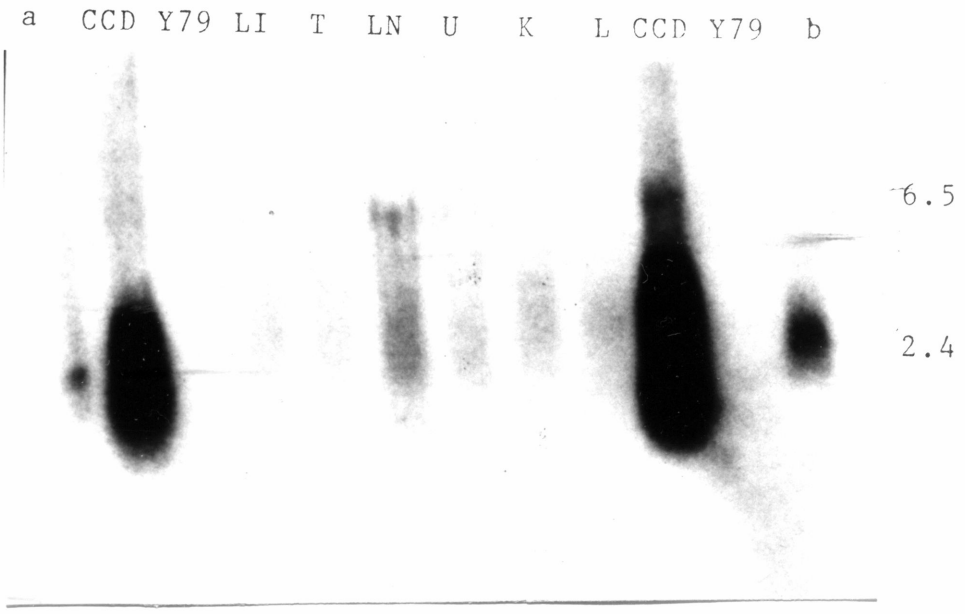


Fig.10. Northern blot analysis of normal canine tissues. After electrophoresis (Fig.5), membrane was hybridized with 32P labelled 0.9R then processed for autoradiography. Lanes a and b contain RNA size markers.

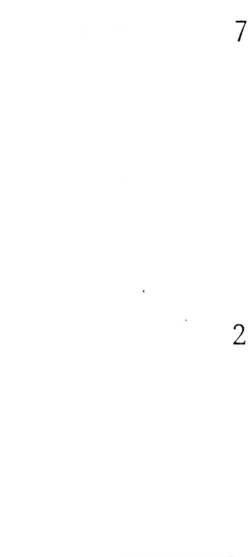


Fig.11. Northern blot analysis of OS tumor. After electrophoresis (Fig.8),membrane was hybridized with 32P labelled 0.9R then processed for autoradiography.

Table 1. Comparison of osteosarcoma in canines and humans. (21)

| | Human | Canine |
|---|---|---|
| INCIDENCE | Annual rate of 1.1/100,000 (9) | Annual rate of 7.9/100,000 (9) |
| AGE | Bimodal peaks; major peak 10-25 yrs; minor peak 50-60 yrs (superimposed on Paget's disease). (5) | Major peak 6-9 yrs (40-52 yrs human equivalent) with a minor peak at 1-2 yrs (12-24 yrs human equivalent). (5) |
| GENETIC FACTORS | Occasional familial tendencies. Higher incidence as a second tumor in retinoblastoma patients. (12) | Familial tendencies in certain large and giant breeds. (4) |
| SITE | 75% in long bones (8) | 77% in long bones (4) |
| CLINICAL FINDINGS | Pain and swelling. Rapidly progressive with early lung metastases. (8) | Same (4) |
| RADIOLOGICAL FEATURES | Aggressive bone lesion including osteolytic and osteoblastic changes (8) | Same (4) |
| HISTOPATHOLOGICAL FEATURES | Presence of sarcomatous tumor and direct formation of tumor osteoid. (8) | Same (4) |
| THERAPY | Surgical excision and chemotherapy. | Same |

## TABLE 2: CANINE OSTEOSARCOMA SAMPLES

| Lane Number | Number | Breed | Age (yrs) | 260/280 Ratio | Condition | mcg RNA | Message Size (kb) |
|---|---|---|---|---|---|---|---|
| 1 | 107014 | Mix | 9 | 1.777 | Intact | 33.43 | Pending |
| 2 | 106684 | Lab Ret. | 2 | 1.824 | Intact | 47.58 | Pending |
| 3 | 106685 | Aust. Shepherd | 14 | 1.397 | N/A | 65.59 | Pending |
| 4 | 106463 | Lab Ret. | 9 | 1.356 | N/A | 89.91 | Pending |
| 5 | 106981 | Mix | 6 | 1.412 | N/A | 31.68 | Pending |
| 6 | 089053 | Gold. Retr. | 5 | 1.760 | Degraded | 16.63 | Pending |
| 7 | 106899-A | Malamute | 9 | 1.714 | Degraded | 47.62 | Pending |
| 8 | 106899-B | Malamute | 9 | 1.219 | N/A | 53.43 | Pending |
| 9 | 104527 | G. Shepherd | 13 | 1.704 | N/A | 50.42 | Pending |
| 10 | 107393 | Mix | 7 | 2.170 | Intact | 9.158 | 7.5, 2.5 |
| 11 | 098428 | Doberman | 7 | 1,273 | N/A | 187.30 | Pending |
| 12 | 104806 | Doberman | 9 | 2.010 | N/A | 22.42 | Pending |
| 13 | 088748 | Gold. Retr. | 14 | 1.781 | Intact | 40.84 | Pending |
| 14 | 105396 | Gold. Retr. | 2 | 1.709 | Intact | 222.52 | Pending |
| 15 | 103573 | Unknown | 4 | 1.448 | Intact | 127.99 | Pending |
| 16 | 107883 | Irish Wolf | 7 | 2.043 | Degraded | 14.27 | Pending |
| 17 | 091091 | Irish Wolf | 4 | 1.650 | N/A | 9.90 | Pending |
| 18 | 107096 | Irish Setter | N/A | 1.600 | Intact | 113.18 | Pending |
| 19 | 107274 | Gold. Retr. | 9 | 1.749 | N/A | 36.66 | Pending |
| 20 | 107651 | Irish Wolf | 8 | 1.823 | Intact | 40.27 | Pending |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 21 | 103955 | Mix | 5 | 2.653 | Intact | 22.99 | Pending |
| 22 | 105989 | Doberman | 11 | 1.440 | N/A | 42.18 | Pending |
| 23 | 099580 | Mix | 9 | 2.650 | Intact | 5.913 | Pending |
| 24 | 0S2.3 | Doberman | 9 | 1.709 | Intact | 109.47 | Pending |
| 25 | 0S2.4 | Gold. Ret | 5 | 1.731 | Degraded | 11.24 | Pending |
| 26 | 0S1.9 | Mix | 3 | 1.726 | Intact | 70.44 | Pending |
| 27 | 0S2.8 | Bulldog | N/A | 1.899 | Intact | 14.29 | Pending |
| 28 | 0S2.7 | Lab Ret. | N/A | 1.067 | N/A | 34.22 | Pending |
| 29 | 0S988 | Borzoi | N/A | 1.402 | Degraded | 14.417 | Pending |
| 30 | 107765 | Mix | N/A | 1.799 | Intact | 36.35 | Pending |
| 31 | 105899 | Unknown | N/A | 1.810 | Intact (?) | 57.44 | Pending |

## TABLE 3: CONTROL SAMPLES

| Sample | Origin | 260/280 | Condition | mcg RNA | Message Size (kb) |
|---|---|---|---|---|---|
| CCD45-7 (CCD) | Human Fibroblasts | 1.926 | N/A | 1.86 | 6.5, 2.4 |
| CCD45-8 | Human Fibroblasts | 1.151 | Intact | 3.11 | 6.5, 2.4 |
| CCD45-15 | Human Fibroblasts | 1.629 | Intact | 25.61 | |
| Y79-5 | Human Rb Cells | 2.083 | N/A | 11.87 | |
| Y79-8 | Human Rb Cells | 1.568 | N/A | 5.24 | |
| Y79-11 | Human Rb Cells | 1.427 | Intact | 27.96 | |
| LI-6 | Liver | 1.744 | Intact | 37.45 | 6.5, 2.4 |
| LI-7 | Liver | 1.728 | Intact | 247.3 | |
| T-1 | Testes | 1.808 | Intact | 13.06 | 2.4 |
| T-2 | Testes | 1.77 | Intact | 211.6 | |
| LN-4 | Lymph Node | 1.941 | Intact | 62.32 | 6.5, 2.4 |
| LN-5 | Lymph Node | 1.71 | Intact | 170.9 | |
| U-5 | Uterus | 1.824 | Intact | 44.06 | 6.5, 2.4 |
| U-6 | Uterus | 1.684 | N/A | 146.8 | |
| K-6 | Kidney | 1.812 | Intact | 11.83 | 2.4 |
| K-7 | Kidney | 1.850 | Intact | 132.8 | |
| L-2 | Lung | 1.561 | N/A | 9.54 | 2.4 |
| L-3 | Lung | 1.619 | Intact | 69.26 | |

## REFERENCES


1. Bech-Nielsen, S. et.al. "Frquency of Osteosarcoma among First-Degree Relatives of St. Bernard Dogs." Journal of the National Cancer Institute. 60:349-353. 1978.

2. Benedict, William, Yuen-Kai Fung and A. Linn Murphree. "The Gene Responsible for the Development of Retinoblastoma and Osteosarcoma." Cancer. 62:1691-1694. 1988.

3. Bookstein, Robert, et al. "Suppression of Tumorigenicity of Human Prostate Carcinoma Cells by Replacing a Mutated RB Gene." Science. 247:712-715. February 9, 1990.

4. Brodey, R.S. "The Use of Naturally Occurring Cancer in Domestic Animals for Research into Human Cancer: General Considerations and a Review of Canine Skeletal Osteosarcoma." Yale Journal of Biological Medicine. 52:345-361. 1979.

5. Brody, R.S. and W.H. Riser. "Canine Osteosarcoma." Clinical Orthopaedics. 12:307-312. 1976.

6. Cavenee, W. K., and M. F. Hansen. "Molecular Genetics of Human Familial Cancer." Cold Spring Harbor Symposia on Quantitative Biology. 51:829-835. 1986.

7. Cavenee, Webster, Alex Koufos and Marc Hansen. "Recessive Mutant Genes Predisposing to Human Cancer." Mutation Research. 168:3-14. 1986.

8. Dahlin, D.C. "Osteosarcoma." In Bone Tumors. 3rd ed. Charles C. Thomas. Springfield, Illinois. pp.226-260. 1981.

9. Dorn, D.R. "Epidemiology of Canine and Feline Tumors." J. Amer. Animal Hosp. Assn. 12:307-312. 1976.

10. Dryja, Thaddeus et. al. "Chromosome 13 Homozygosity in Osteosarcoma Without Retinoblastoma." American Journal of Human Genetics. 38:59-66. 1988 .

11. Friend, Stephen et.al. "A Human DNA Segment with Properties of the Gene That Predisposes to Retinoblastoma and Osteosarcoma." Nature. 323:643-646. October 16, 1986.

12. Goorin, A.M. at.al. "Osteosarcoma: Fifteen Years Later." New England Journal of Medicine. 313:1637-11643. 1985.

13. Green, Michael. "When the Products of Oncogenes and Anti-Oncogenes Meet." Cell. 56:1-3. January 13, 1989.

14. Haines, Deborah and Oyvind Sverre Bruland. "Immunohistochemical Detection of Osteosarcoma-Associated Antigen in Canine Osteosarcoma." Anticancer Research. 9:903-908. 1989.

15. Hansen, Marc et.al. "Osteosarcoma and Retinoblastoma: A Shared Chromosomal Mechanism Revealing Recessive Predisposition." Proceedings of the National Academy of Science, USA. 82:6216-6220. September 1985.

16. Hansen, Marc and Webster Cavenee. "Tumor Suppressors: Recessive Mutations that Lead to Cancer." Cell. 53:172-173. April 22, 1988.

17. Huang, Huei-Jin Su et al. "Suppression of the Neoplastic Phenotype by Replacement of the RB Gene in Human Cancer Cells." Science. 242:1563-1566. December 16, 1988.

18. Hunter, Tony. "The Proteins of Oncogenes." Scientific American. 251(2):70-79. August 1984.

19. Knudson, Alfred G. "Mutation and Cancer: A Statistical Study of Retinoblastoma." Proceedings of the National Academy of Science, USA. 68(4):820-823. April 1971.

20. Knudson, Alfred G. "Retinoblastoma: A Prototypic Hereditary Neoplasm."

Seminars in Oncology. 5(1):57-60. March 1978.

21. Kochevar,D.T., Kochevar, J. and Garrett, L. "Low Level Amplification of c-*sis* and c-*myc* in a Spontaneeous Osteosarcoma Model." Cancer Letters. 53:213-222. 1990.

22. Lee, Eva et.al. "Molecular Mechanism of Retinoblastoma Gene Inactivation in Retinoblastoma Cell Line Y79." Proceedings of the National Academy of Science, USA. 85:6017-6021. August 1988.

23. Maniatis, Fritsch, and Sambrook. Molecular Cloning: A Laboratory Manual. 2 ed. vol.2.

24. McFall, Rosemary et.al. "Characterization of a New Continuous Cell Line Derived from a Human Retinoblastoma." Cancer Research. 37:1003-1010. April 1977.

25. Murphree, A. Linn and William F. Benedict. "Retinoblastoma: Clues to Human Oncogenesis." Science. 223:1028-1033. March 9, 1984.

26. Weinberg, Robert. "A Molecular Basis of Cancer." Scientific American. 249:126-141. November 1983.

27. Weinberg, Robert. " Finding the Anti-Oncogene." Scientific American. September 1988.