GENETIC ALTERATIONS IN PEDIATRIC GERM CELL TUMORS

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Kimberly Jane Bussey

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CERTIFICATE OF APPROVAL

This is to certify that the Ph.D. Thesis of

Kimberly J. Bussey

has been approved

[redacted]
Professor in charge of thesis

[redacted]
Professor in charge of thesis

[redacted]
Member

[redacted]
Member

[redacted]
Member

[redacted]
Associate Dean for Graduate Studies

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Abstract

The chromosomes of 81 pediatric germ cell tumors (GCT) were analyzed as part of two clinical treatment trials, INT-0098 and INT-0097, conducted by the Children's Cancer Group. This study comprises the largest group of pediatric GCTs examined to date, allowing novel observations dividing the tumors into six groups with possibly different etiologies based on their chromosomal profile in conjunction with patient age, sex, tumor location and histology. The most common abnormalities seen differed for each group. The gain of an isochromosome 12p, common in adult testicular GCTs, was frequent only in the tumors from adolescent and older boys. Deletion of 1p/gain of 1q and +3 were the most common abnormalities among the malignant tumors from both sexes.

The incidence of chromosome 1p loss and 12p amplification was explored by interphase FISH in 53 pediatric GCTs that had been previously G-banded. Twelve of 53 tumors were identified with a loss of 1p36. Nine tumors were confirmed to be malignant, yielding a significant association of 1p loss with malignancy in pediatric GCTs ($p=0.00115$). Five of 18 tumors from male patients had amplification of 12p, consistent with G-band results. Combined analysis of our data with the literature revealed a significant correlation of 12p amplification with patient age ($p=0.000196$). Amplification of 12p was only seen in 1 of 35 tumors from female patients. Five female GCTs had numerical abnormalities of chromosome 12, and 2 tumors showed complete lack of 12p. This spectrum of abnormalities differs from what is seen in the male tumors, providing further evidence for different etiologies of GCTs between the sexes.

To explore the timing of imprint switching in the human germ line, the methylation status of *SNRPN* in DNA from the cultured cells of 30 pediatric GCTs was examined by Southern blot using an *Xba*I/*Not*I dual digestion. The results were compared to the predicted methylation pattern for the hypothesized primordial germ cell (PGC) giving rise to the

tumor. Eighteen of the 30 tumors (6/7 sacrococcygeal tumors, 9/11 ovarian tumors, 1/3 testicular and 2/9 extragonadal pediatric GCTs) had results matching the predictions. The data support a similar pattern of demethylation and remethylation of *SNRPN* in the germ line between mice and humans. The data also indicate multiple etiologies for extragonadal GCTs in children and begin to suggest a time frame for the initial events in GCT tumorigenesis.

To further support a time frame for the initial events leading to germ cell tumor formation, the methylation pattern of *SNRPN* was examined in frozen tissue samples of 35 adult male GCTs and 20 pediatric GCTs from various locations. Twenty-three of 35 adult male GCTs showed evidence of germ cell origin. Among the 20 pediatric GCTs, 16 tumors (4 of 5 tumors from sacral/retroperitoneal locations, 11 of 14 ovarian GCTs and 1 of 2 extragonadal GCTs) also demonstrated evidence for germ cell origin. The data continue to support the model of imprint erasure and resetting established in the mouse, and suggest the events leading to GCT formation act on PGCs as early in embryonic development as 24 days post conception.

Introduction

Statement of the Problem

Pediatric germ cell tumors (GCTs) are rare malignancies, accounting for less than 3% of childhood cancer (Young and Miller 1975). Because of their rarity, it has been difficult to accumulate information about the genetic changes characterizing these tumors and their influence on tumor behavior. Treatments for pediatric GCTs have been based on those devised for the adult counterparts and use surgery combined with multi-agent chemotherapy including platinum (Castleberry et al. 1997). Such treatments result in a 90% cure rate for men with newly diagnosed testicular GCT (Murty and Chaganti 1998), and have improved survival in the pediatric population. However, childhood GCTs follow a different clinical course when compared to the adult counterparts (Harms and Jänig 1986). While pediatric GCTs have a good prognosis in the majority of cases, they can be difficult to manage because of a lack of accurate predictors of tumor behavior (Castleberry et al. 1997). The goal in treatment is to minimize permanent toxicity and preserve future fertility while still maintaining high response rates and good long-term disease free survival. We believe that by characterizing the genetic abnormalities of a large series of these tumors, we might be able to delineate subgroups which have a different etiologies and therefore possibly different clinical courses.

Cancer Cytogenetics: A Historical Perspective

The Boveri Hypothesis

Cancer is the term applied to over 1000 different diseases that share the common feature of unregulated cell growth leading to tumor formation. Morbidity and mortality result as the cancer disrupts the functioning of surrounding normal tissue, both at the primary and distant sites. Cancer biology has embraced the paradigm that cancer is a disease of genes. This view was first put forth by Theodor Boveri and summarized in his work, The Origins of Malignant Tumors (1929). The central tenet of his thesis is that an abnormal chromatin

complex causes an irreparable defect within the cell resulting in uncontrolled cell growth. He based his theory on the following assumptions: 1) the defect causing cancer must reside within the cell; 2) the nucleus must be the site of the defect; 3) unlimited cell growth can result from alterations of a cell's interaction with its environment; and 4) different cellular qualities belong to different chromosomes, such that loss or gain of chromosomes or pieces of chromosomes results in abnormal cellular metabolism and response to the environment. He states the following (Boveri 1929, p. 113):

> The chief thing, to be sure, is hypothetical, that is, the question whether an abnormal chromosome-complex can arise of such a kind that it can force the cells which possess it to continue to multiply. This assumption must be made ad hoc. But much is to be said in its favor. Above all I hold it to be without any doubt that the tendency to continued multiplication is a primordial quality of cells, which only secondarily becomes inhibited in many-celled organisms through environmental influences. That the cells conform to this inhibiting action as a rule, and only in response to definite changes in their surroundings follow once more their original tendency to divide, presupposes a cellular apparatus especially sensitive to the surrounding conditions. If such is present we can conceive of disturbance in it, which would make it lose its sensitiveness to the surrounding conditions. Thus the inherent impetus of the cell to multiply would be set free and would follow its course without regard to the needs of the rest of the body.
>
> We seem to be justified in placing the assumed regulatory arrangement in the nucleus, because there certainly exist relations between the chromatin and the regulation of division. Thus definite changes in the constitution of the nucleus might present disturbances through which the power of reaction to the conditions of normal surroundings are lost.

With this as his central concept, Boveri then went on to predict what might be the consequences of such a model; many of these predictions have been proven and are now accepted as fact. Boveri believed that there was a balance between "inhibiting" and "promoting" chromosomes for cell division and growth. Disruption of this balance leads to uncontrolled cell division. Cancer is now viewed as the synergistic outcome of the loss of

function of tumor suppressor genes (inhibitors) and the over-expression of oncogenes (promoters).

Secondly, Boveri postulated that tumors became more malignant as their chromatin content became more abnormal. Work on several cancers, including breast, colon, and prostate, confirm that increasing malignancy is correlated with increasing numbers of mutations, whether at the gene or chromosome level. Boveri also believed the concept of tumors arising from a single cell, clonality, was an inescapable outcome of his model (Boveri 1929, p. 39):

> It is very striking that, although different kinds of tumors may come from the same mother-tissue, every single tumor has, typically, a uniform character, which in general continues to exist in the metastases and transplantations. This appearance depends, as no one doubts, on the fact that wherever a tumor may spread, all of its cells arise from the tumor Anlage. Now if we ask "how do the cells of young primary tumors which are barely visible acquire the uniform type" the answer must always be, "because they too go back to common ancestors which also had the same abnormal constitution." Following out these considerations, would lead to the conclusion that typically every tumor arises from a single cell....
>
> This primordial cell of a tumor, as I shall call it in what follows, is according to my theory a cell which contains, as a result of an abnormal process, a definite and wrongly combined chromosome-complex. This is above all the cause of the tendency to rapid cell proliferation, which is passed on to all of the descendants of the primordial cell, as far as they arise by regular mitotic cell division. But also all other abnormal qualities, which the tumor shows, are involved in the abnormal chromosome combination of the primordial cell and are inherited by all of its descendants, as long as subsequent cell-divisions take place by normal bi-polar mitosis.

Boveri favored the occurrence of multi-polar mitoses as a way to generate the chromatin imbalance in cancer cells. Indeed, recent work (Fukasawa et al. 1996) has demonstrated aberrant centrosome replication in cells with mutant p53, one of the most widely studied tumor suppressors, leading to abnormal chromosome segregation. Lastly, Boveri

hypothesized that different abnormal chromatin contents would lead to different types of tumors. It is this last prediction of his model that underlies the study of genetic changes in tumorigenesis today. Although Boveri's ideas form the current basis for modern cancer genetics, it has not been until recently that his work and his ideas could be experimentally supported.

Oncogenes and Tumor Suppressors

Boveri's ideas of promoting and inhibiting factors that reside on chromosomes has been shown experimentally in the form of proto-oncogenes and tumor suppressor genes, respectively. Oncogenes were initially discovered as part of retroviruses that could cause tumor formation in a variety of model systems, including chickens (Rous 1911). These genes were discovered to have a cellular counterpart. The mode of replication of the viruses enabled them to pick up the cellular genes and make them part of their genome. The mode of action of oncogenes is one of promotion, usually by being expressed at either an inappropriate time and/or at higher levels than normal. Cytogenetic evidence for oncogene amplification has come in the form of double minute chromosomes or homogenously staining regions.

Tumor suppressor genes were more difficult to identify. Knudson's study of retinoblastoma led to the two-hit hypothesis (1971). He noted that in familial cases, the onset of retinoblastoma was at a younger age and often involved bilateral presentations. He postulated that a germ line mutation predisposed an individual to form tumors, but it was not sufficient to do so. A second, somatic mutation was necessary for tumor development. The chances of such a mutation happening in one cell of the retina were such that this hypothesis could explain the dominant inheritance pattern in the familial cases. Such a gene was then considered to be a suppressor of tumor formation. Cytogenetic analysis of the peripheral blood of familial retinoblastoma cases led to the localization of this gene to 13q14 (Francke 1976). Somatic cell fusion experiments using human tumor cell lines and normal human

fibroblasts confirmed that the malignant phenotype was recessive and allowed the observation that loss of certain chromosomes restored a malignant phenotype, helping to identify chromosomes that harbor tumor suppressor genes (Stanbridge et al. 1982).

Technical Advances

Boveri derived his view of cancer from his experimental work on sea urchin eggs, as well as the observations of abnormal nuclear figures reported by von Hansemann in 1890. At the time, the only way to observe the genetic changes in a cell was by the use of paraffin-embedded sections. Such material is ideal for preserving tissue architecture but is almost impossible to use for any detailed study of mitotic figures. The material is so difficult to work with that for the first half of the 20th century, the chromosome count of humans was thought to be either 47 or 48, depending on the investigator. Several technical advances occurred which permitted the development of modern cancer cytogenetics (for an extensive review, see Hsu 1979).

Improving Chromosome Morphology

One obstacle to overcome was the poor morphology of the chromosomes. In paraffin-embedded material, mitotic figures are often cramped and overlapped, with little evidence of individual chromosomes. Furthermore, most mitotic figures are not in metaphase. Three advances served to improve this. The addition of colchicine or its derivative, Colcemid, arrests cells at metaphase (Levan 1938) by disrupting the actin spindle apparatus. This serves to increase the number of mitotic figures present in a sample at a stage that is useful for analysis. The next technical improvement was the discovery, or rediscovery, that exposing tissues to a hypotonic solution would swell the cell and allow for chromosome spreading (Slifer 1934; Hsu 1952; Hughes 1952; Hsu and Pomerat 1953). The third advance was borrowed from plant and fly cytogeneticists. The discovery that mammalian tissues could be prepared by a squash method was a boon to early cytogeneticists (Makino and Nishimura 1952). Sectioning of tissues invariably leads to the bisection of nuclei,

which makes it very difficult to get an accurate representation of chromosome morphology, even when serial sections are used. In addition, the three-dimensional nature of paraffin-embedded tissue requires constant adjustment to the focal plane during observation. This compounds the problem of determining the morphology of chromosomes. The squash method flattens cells so they are visible in a single plane of focus. The combination of arresting cells in metaphase with using a hypotonic solution and a squash preparation (later replaced by air drying which accomplishes the same result with less work) resulted in the detailed examination of reproducible chromosome morphology in the human.

Tissue Culture

Concurrent with the developments in sample preparation, the in vitro culture of cells from various tissues was developed. This was a benefit because cells in tissue culture grow either in suspension or in monolayers, depending on the tissue. Such growth patterns allow nearly single cell suspensions to be made, which can then be easily applied to slides for observation. Two problems had to be overcome. First, single cells had to be cultured in such a manner as to give rise to entire populations, and secondly, these populations had to be viable over long periods with actively dividing cells. The viability issue was addressed in 1912 by Carrel. He cultured an explant of chick muscle and, through feeding, was able to keep it viable long after the chicken itself had died. The medium of the day was usually a buffered salt solution to which serum or embryo extract was added. It was not chemically defined, and cell growth varied from tissue to tissue, with single cells unable to grow in large volumes of medium. In order to optimize cell culture medium, Eagle (1955) demonstrated that cells needed 13 amino acids in the L-conformation, seven vitamins, certain salts, and glucose, plus as little as 1% dialyzed serum. The presence of serum protein was necessary for successful cell growth (1955). Work by Sanford et al. (1948) showed that cells conditioned the medium in which they were grown and providing the appropriate cell to medium ratio permits the growth of single cells. To obviate the requirement for large

numbers of cells, Puck and Marcus (1955) employed an irradiated feeder layer to condition the medium and allow small numbers of cells on a suspended coverslip to grow and divide. The discovery of the mitogenic properties of phytohemmagglutinin on circulating peripheral lymphocytes marked another step forward (Nowell 1960). The methodologies for cell culture allowed the correct chromosome number in man to be determined from studies performed on cells derived from embryonic lung biopsies (Tijo and Levan 1956).

The application of cell culture to malignant tissues has been difficult. First of all, in vitro cell culture has been optimized for the growth of normal cells. A large force behind the advances in cell culture was the desired to have normal cells that could be engineered to produce products like viral particles for vaccines (Spier and Griffiths 1985). Because of that, cell culture is designed to simulate the tissue environment of normal cells. Most of the early studies employing malignant material were from metastatic tumors that were highly aggressive and had minimal requirements, and therefore could be grown in minimal culture medium. A great number of primary solid tumors do not fare as well in vitro. In 1952, Gey and collegues reported on their attempts to grow material in vitro from various biopsies of normal and neoplastic tissues from the same cervix. They were only able to establish one line, growing it in a complex medium of human placental cord serum, bovine embryo extract, and chicken plasma. Their attempt illustrates the continued problem of solid tumor cytogenetics. While tumor cells may have altered growth kinetics that permit them to grow in vivo, when the selective pressures change, they may not survive as well as their normal counterparts. Several possibilities may explain the failure of primary tumors to grow in culture including the absence of critical growth factors or other mitogenic stimulus, a small number of tumor cells initially present in the culture, and/or altered cell cycle kinetics.

Chromosome Banding

Early studies of chromosomes in cancer demonstrated that aneuploidy was common, and that more advanced cancers had increasing levels of chromosomal abnormalities. However,

prior to the advent of banding techniques, it was difficult to establish that chromosome changes per se were nonrandomly involved in cancer. Without banding, identification of non-random chromosome abnormalities in tumors required changes in chromosome morphology that were distinct, either by size and/or centromere placement. Hungerford and Nowell (1960) were the first to show a specific chromosome abnormality was associated with a particular cancer, chronic myelogenous leukemia (CML). They saw that one of the G-group chromosomes was much smaller than the others. It became known as the Philadelphia chromosome (Ph). The discovery that quinacrine mustard could be used to give a reproducible, characteristic banding pattern for each individual chromosome (Caspersson et al. 1970), followed shortly by the discovery of G- and R-banding (Dutrillaux and Lejeune 1971; Seabright 1971), meant that by 1973 , Janet Rowley could determine that the Ph-chromosome was really the derivative chromosome 22 in a reciprocal translocation between the long arms of chromosomes 9 and 22. This is now known as the Philadelphia rearrangement or t(9;22)(q34;q11). Other diagnostic and/or prognostic findings found include t(8;14)(q24;q32) in B-cell lymphomas, particularly Burkitt's lymphoma and t(X;18)(p11;q11) in synovial sarcoma (Heim and Mitelman 1995).

The outgrowth of normal cells results in a high "normal" rate for solid tumor karyotypes. It is much easier to culture leukemias and lymphomas; hence, the significant difference in numbers between cytogenetically characterized leukemias and equally characterized solid tumors (see Heim and Mitelman 1995 for review). Successful culture of solid tumors requires a great deal of time and patience to accumulate sufficient cells in metaphase to permit a study, often by using a variety of culture methods and short culture times to capture the abnormal clone. Such techniques have enabled the detection of eight distinct cytogenetic subgroups in breast cancer, a particularly difficult tumor to grow in culture (Pandis et al. 1995).

Cancers, particularly solid tumors, may have highly rearranged chromosomes, and many are unidentifiable even with banding techniques. Thus, the next technical revolution that

forwarded the field of cancer cytogenetics was the development of sensitive *in situ* hybridization techniques.

In Situ *Hybridization*

In situ hybridization of nucleic acids to cytological preparations began in the late 1960's with the use of ^{3}H-labeled RNA. In 1969, Gall and Pardue hybridized ^{3}H-labeled rRNA from *Xenopus* to cytological preparation of *Xenopus* oocytes. They demonstrated a similar technique could be employed with DNA to localize certain fractions to specific places in the cell (Pardue and Gall 1969). By the following year, Pardue and Gall reported successful hybridization of both radioactive satellite DNA and RNA transcribed from satellite DNA in vitro to metaphase spreads of the mouse, localizing this DNA fraction to the centromeric heterochromatin (1970). Hybridization to cytological preparations required a higher specific activity as well as a longer exposure when compared to hybridization to "naked" DNA immobilized on a solid support. The experiments of Pardue and Gall (1970) required exposure times of days with RNA and months with DNA. As a result, the first hybridizations were done with probes that were abundant, such as satellite DNAs. As *in situ* hybridization techniques and the isolation of small portions of unique sequences of DNA improved, it was possible to map single genes. Gerhard et al. (1981) reported the hybridization of the human α-globin gene cluster to chromosome 16. That same year, Harper and Saunders (1981) published a method to hybridize unique sequences to G-banded chromosomes. However, such techniques required that many metaphases be observed and a plot of grain distribution over each chromosome be created in order to determine the most likely location of a probe. The paucity and mediocre qualitity of metaphases in most cancers made this technique unfeasable. Additionally, the necessity of several days for exposure made the technique time intensive.

In an effort to overcome the disadvantages of working with radioactive probes, several investigators tried using fluorescence. Cheung et al. (1977) used mRNA attached to highly

fluorescent latex microspheres to map the α- and ß- globin genes. The use of fluorochrome labeled RNA for *in situ* hybridization to Drosophilia was reported in 1981 (Bauman et al.). The development of hapten-labeled nucleotides (Langer et al. 1981) enabled the hybridization of satellite DNA using biotin-labeled probes reported by Manuelidis et al. in 1982 . While fluorescent methods were faster than radioactive methods, problems existed with the visualization of unique sequences. In fluorescent *in situ* hybridization, the ability to visualize a probe is related to the size of the target. Probes smaller than 10 kb are difficult to see and distinguish from background fluorescence. The advent of reliable methods to reduce signal to noise ratio resulted in a wide acceptance of the technique. By the early 1990's, fluorescent *in situ* hybridization (FISH) was being routinely used to further characterize the chromosome abnormalities in tumor cells. The development of degenerate oligonucleotide primed polymerase chain reaction (DOP-PCR) (Telenius et al. 1992) coupled with the microdissection of material directly from cytogenetic preparations allowed researchers to begin to identify the chromosomal origin of marker chromosomes, heterogeneously staining regions, and double minutes (Xu et al. 1995). Recently, differential labeling techniques have been applied to yield whole chromosome paint probes with different spectral profiles. These "chromosome paints" have been used with computer aided image acquisition to yield M-FISH (Speicher et al. 1996) or SKY (Schrock et al. 1996; Macville et al. 1997) (depending on how the image is acquired), a method for painting all of the chromosomes simultaneously. The technique is particularly powerful in identifying cryptic translocations (Veldman et al. 1997; Allen et al. 1998; Tosi et al. 1999).

These techniques all rely on the presence of metaphase spreads. However, as mentioned, many cancers yield few metaphases. Prior to the introduction of FISH, the low number of metaphase spreads limited investigators to evaluating ploidy levels via DNA content measurements or investigating specific genomic rearrangements with molecular methods. The FISH technology spawned methods for dealing with a low mitotic index. One method is interphase FISH. Although whole chromosome paints appear diffuse in interphase,

reflecting the de-condensed nature of the chromosomes, smaller probes recognizing targets such as chromosome-specific satellite DNA or unique sequence DNA give distinct signals. Such signals can be counted, and indeed numerous papers have used chromosome specific alpha-satellite DNA to determine the number of homologs of a given chromosome present in a tumor. Another method is comparative genomic hybridization or CGH (Kallioniemi et al. 1992). This technique involves the hybridization of the DNA from karyotypically normal cells labeled in one color (red), and the DNA of the test cells labeled in a different color (green), to the metaphase spreads of a chromosomally normal male. The relative intensities of the two fluors are measured along the length of each chromosome in several metaphases after image aquisition and computer analysis. A graph is produced showing the relative gains and losses along each chromosome. Where the test DNA and the reference DNA are equal in copy number, the signal is yellow. When there is an over-representation of test DNA, a green signal is produced, while an under-representation of test DNA gives a red signal. The technique is good for detecting net gains or losses, but gives no information about the structural context of those abnormalities. It also fails to recognize important balanced rearrangements, such as the t(9;22)(q34;q11).

Chromosome Abnormalities

It is the structural context of chromosome abnormalities that has been the most fruitful, as well as most frustrating, for cancer cytogeneticists. Tumors demonstrate a wide range of genomic derrangement, including both numerical changes, such as ploidy problems or specific trisomies or monosomies, and structural rearrangements, including translocations, inversions, deletions, additions, and gene amplification. The mechanism(s) leading to these changes is still an enigma. Recurrent reciprocal translocations seem to be the easiest to explain because they often result in the creation of an oncogenic fusion gene when the breakpoints of the two chromosomes are joined. The creation of the fusion gene then leads to a growth advantage and tumor formation in susceptible tissues. Alterations in ploidy that

are not simply doubling or halving of the diploid chromosome number can best be explained by multi-polar mitosis, a common phenomenon in cells lacking p53 function (Fukasawa et al. 1996). The complex rearrangements leading to unbalanced additions and deletions of chromosomal material has been shown to be caused in some cases by cycles of breakage-fusion-bridge events (D. Gisselsson, unpublished), first reported by McClintock in maize (1941). Variables such as chromatin structure at specific loci, viral or mtDNA integration sites, fragile sites, evolutionarily conserved breakpoints, enzymatic cleavage sites, and repeat DNA sequences are all being explored for possible roles in generating non-random structural changes in tumors (E. Schröck, unpublished). However such abnormalities arise, the common feature is that for any given tumor type, there is a pattern of non-random chromosome abnormalities which confers a selective advantage to the tumor over the normal tissue, enabling it to grow and metastasize. The challenge for the cancer cytogeneticist is to elucidate the pattern of non-random changes in the background of secondary, random events, thought to be indicative of genomic instability in general. While many tumor types have been studied, little information exists on the cytogenetics of pediatric GCTs.

Germ Cell Tumors

The Primordial Germ Cell

The term germ cell tumor (GCT) refers to a group of heterogeneous tumors that are believed to have a common cellular origin, the primordial germ cell (PGC). To understand the presentation of GCTs, it is necessary to understand the biology of the PGC. In humans, the PGCs appear at about 24 days postfertilization in the endodermal layer of the yolk sac near the allantois. They then migrate caudally from the allantois through the hindgut and into the dorsal mesentary until they reach the genital ridge. The control of the migration is not well understood. Expression of c-kit by the PGC and stem cell factor by the surrounding tissue is necessary for germ cell migration and survival (Castleberry et al. 1997). Oct-3/4

expression is also necessary in the germ line to maintain totipotency (Pesce et al. 1998). Other factors, including possible temporal components, remain uncharacterized. Once the PGCs have reached the genital ridges, they undergo a series of mitotic divisions. In the female, meiosis begins at about 3 months gestation and continues until just prior to birth. The oocytes are arrested in meiosis I at dictyotene (a stage similar to the transition between diplotene and diakinesis). They remain in this state until the onset of puberty, when ovulation stimulates the progression through the first meiotic division and into meiosis II, which is not completed unless fertilization occurs. In the male, the germ cells remain quiescent until puberty when mitosis resumes in the premeiotic germ cells and meiosis begins. Once meiosis starts in a male germ cell, it continues without interruption.

Epidemiology

Germ cell tumors present in humans at both gonadal and extragonadal locations, usually along the midline. Almost one-half to 2/3 of GCTs in children are extragonadal (Harms and Jänig 1986). There is a bimodal distribution in the pediatric population, with a peak in children under 3 years old and a second peak in children over 12 (Dehner 1983). Within this distribution, there is a predominance of females in the first three years of life with sacrococcygeal teratomas, while testicular GCTs account for the greater number of males in adolescence. Overall, there is almost a 2:1 ratio of female to male patients in the pediatric age range, attributed to the earlier development of ovarian GCTs in adolescent girls combined with the greater numbers of sacrococcygeal tumors among girls as compared to boys (Dehner 1983). The presentation of the tumors can be benign and/or malignant, and can resemble germ cells (seminomas, dysgerminomas, and germinomas), or differentiate through either embryonic (mature or immature teratomas) or extraembryonic (embryonal carcinoma, endodermal sinus tumors/ yolk sac tumors, or choriocarcinoma) pathways (Castleberry et al. 1997). GCTs can also present in adulthood, though the frequency of specific histologic types, anatomical location, clinical behavior, and natural history of GCTs

differ between children and adults (Harms and Jänig 1986).

Risk Factors

Several risk factors have been explored for possible association with GCT formation. Those that have shown association include: 1) gonadal dysgenesis, particularly related to constitutional chromosome abnormalities. Studies have reported an increased incidence of GCTs in patients with XXY Klinefelter syndrome, trisomy 21, XY females and 45,X/46,XY females (Scully 1970; Schellhas 1974; Sogge et al. 1979; Curry et al. 1981; Turner et al. 1981; Mann et al. 1983; Muller et al. 1985; Nichols et al. 1987; Hasle et al. 1992; Derenoncourt et al. 1995; Dieckmann et al. 1997; Satge et al. 1998); 2) cryptorchidism; 3) maternal exogenous hormonal use during pregnancy; 4) radiation exposure; and 5) childhood viral infections (e.g. mumps) (Shu et al. 1993).

Genetic Changes

Most of the information about the genetic changes in GCT comes from the study of adult testicular cancer. These tumors are thought to progress through a series of steps that begins with CIS (carcinoma *in situ*), progresses to seminoma, and then to non-seminomatous tumors (de Jong et al. 1990). Concurrent with this progression is a series of karyotypic changes. Ploidy analysis shows CIS and seminomas are usually near-tetraploid and appear to lose chromosomes to become near-triploid in the non-seminomatous stage (de Jong et al. 1990). The most common finding in adult male GCT is the presence of an isochromosome 12p. Other karyotypic changes include non-random loss of chromosomes 5, 11, 13, and 18, as well as gains of the X chromosome and chromosomes 7, 8, and 12. Structural changes commonly involve 12q, 1p, 1q, 9q, 6q, and 7p (de Jong et al. 1990; de Jong et al. 1997).

Less is known about ovarian GCT in adults. Most commonly, these tumors are benign teratomas with a 46,XX karyotype. About 65% of benign ovarian teratomas are parthenogenetic as a result of failure of meiosis II polar body formation or endoreduplication

of a haploid ovum (Surti et al. 1990). Such tumors are homozygous for centromeric chromosome heteromorphisms and are classified as being (23,X)x2. Simple numerical and/ or structural rearrangements typify the chromosomally abnormal ovarian teratomas. Gains of chromosome 3, 8, 12, and 14 are the most frequent changes seen. Some dysgerminomas resemble the seminomas of the adult testis, including the presence of i(12p) (Atkin and Baker 1987; Speleman et al. 1990; Speleman et al. 1992; Rodriguez et al. 1995; van Echten et al. 1998).

Specific Aims and Hypotheses

Overall Hypothesis

The overall hypothesis for this investigation is as follows: A pattern of non-random cytogenetic and molecular genetic changes exists in pediatric germ cell tumors which identifies distinct subgroups with potentially different etiologies. This hypothesis was approached through four specific aims and accompanying hypotheses. They are described below.

Specific Aim 1

The first aim of this project was to analyze the chromosome aberrations in pediatric germ cell tumors and correlate the chromosomal patterns with known stratifying factors such as patient age, patient sex, and tumor location. We hypothesized that patient age, sex, tumor site, and tumor histopathology are associated with specific patterns of chromosome changes in pediatric germ cell tumors (GCTs). To this end, 81 pediatric germ cell tumors were characterized cytogenetically and the results used to further stratify patients into subgroups with possibly different etiologies.

Specific Aim 2

The second specific aim was to assess the incidence of 1p36 loss, including the midi-satellite (D1Z2) and $p58^{clk-2}$ in pediatric germ cell tumors of various histologic types. We tested the hypothesis that loss of 1p36, as determined by FISH with probes for D1Z2 and $p58^{clk-2}$, is more prevalent in pediatric germ cell tumors with malignant histologic types than those of benign histologic types. Three literature reports showed that deletions of the distal short arm of chromosome 1 are associated with a malignant histology in pediatric germ cell tumors in both gonadal and extragonadal locations (Stock et al. 1994; Jenderny et al. 1995; Jenderny et al. 1996). These investigators used interphase fluorescent *in situ* hybridization (FISH) on paraffin embedded material with probes for D1Z2, a subtelomeric midi-satellite repeat in band 1p36.3 on the distal short arm and the chromosome 1 alpha satellite which is located on the long arm, just below the centromere. They examined the number of D1Z2 and alpha satellite signals. Each probe was hybridized separately on serial sections, and the average number of signals per probe was compared. In tumors in which the distal short arm of chromosome 1 is not deleted, the number of signals for both probes should be equal. When the short arm is deleted, the number of alpha satellite signals will be greater than the number of D1Z2 signals. An additional interphase FISH study on the direct harvests of 10 pediatric endodermal sinus tumors using probes for chromosome 1 alpha satellite and $p58^{clk-2}$, which detects the PITSLRE kinase locus (the name refers to the one letter amino acid designations of the PTSAIRE box regions of kinases related to $p34^{cdc2}$) and also maps to 1p36, detected loss in 8 of 10 tumors (Perlman et al. 1996). It could be postulated that loss of 1p36 is indicative of progression and could serve as a diagnostic marker for malignancy in pediatric germ cell tumors.

Specific Aim 3

Our third specific aim was to assess the incidence of 12p amplification in pediatric germ cell tumors of both sexes. We had two hypotheses: (1) Among males, amplification of 12p is

more common in germ cell tumors from adolescents and adults and (2) 12p amplification is minimally involved in female germ cell tumors.

Amplification of chromosome 12 short arm material, particularly in the form of an isochromosome composed of two short arms joined at the centromere, i(12)(p10), has been identified in 80% of adult testicular tumors (Rodriguez et al. 1992; Rodriguez et al. 1993). Recent evidence suggests amplification of two regions on 12p, 12p11.2-12 and 12p13, play a role in the progression of adult testicular germ cell tumors (Henegariu et al.1998). The lack of i(12)(p10) in the pediatric germ cell tumors in pre-pubertal males suggests that they represent a distinct etiological entity from the adult male germ cell tumors. Failure to detect amplification of the short arm of chromosome 12 by karyotyping does not necessarily preclude the presence of amplification by other means; amplification could take the form of an unrecognizable marker chromosome or an HSR. Additionally, since the karyotype analysis relies on dividing cells, a non-dividing clone with such an abnormality would be missed.

The presence of i(12p) has been observed in five of eight ovarian dysgerminomas in the literature, including one case from our own study (Atkin and Baker 1987; Jenkyn and McCartney 1987; Rodriguez et al. 1995; Bussey et al. 1999). More common is a whole chromosome gain of chromosome 12, particularly in malignant tumors. However, the role of 12p amplification in the pathogenesis of female germ cell tumors remains unclear.

Specific Aim 4

The final specific aim of this project was to assess the methylation pattern of the 5' UTR (untranslated region) of exon 1 of *SNRPN* in pediatric germ cell tumors and attempt to correlate this information with cytogenetic aberrations. This was based on the hypothesis that the pattern of imprint erasure or loss and re-establishment is similar between mice and humans. A corollary of this is that the methylation pattern of the 5' UTR of exon 1 of *SNRPN* is indicative of the stage of development of the cell from which the tumor arose.

This would allow the determination of a temporal interval during which the initiating events leading to tumor formation are acting.

The gene for the small nuclear ribonucleoprotein polypeptide N (*SNRPN*), on chromosome 15, is imprinted in humans and only expressed from the paternal allele. The CpG island in the 5'-untranslated region of the first exon shows stable parent-specific methylation patterns in all somatic tissues tested, with the maternal allele methylated while the paternal allele is unmethylated. It has been demonstrated that the methylation of the maternal allele inhibits transcription (Shemer et al. 1997). The establishment of this pattern and hence the imprinted expression occurs sometime during gametogenesis.

Data from the mouse suggest that the somatic methylation pattern remains intact in primordial germ cells up to the time of colonization of the genital ridge, when all methylation is removed at this locus and expression of *SNRPN* occurs from both parental alleles (Szabo and Mann 1995; Shemer et al. 1997). By the time germ cells in both the male and female have completed gametogenesis, parent-specific methylation has been established; oocytes are methylated while spermatocytes remain unmethylated (Shemer et al. 1997). Recent expression data from the human support this model (Huntriss et al. 1998).

The hypothesis that the pattern of imprint erasure and re-establishment is similar between mice and humans, as evidenced by the methylation of *SNRPN*, leads to the following testable predictions (see figure 1.1):

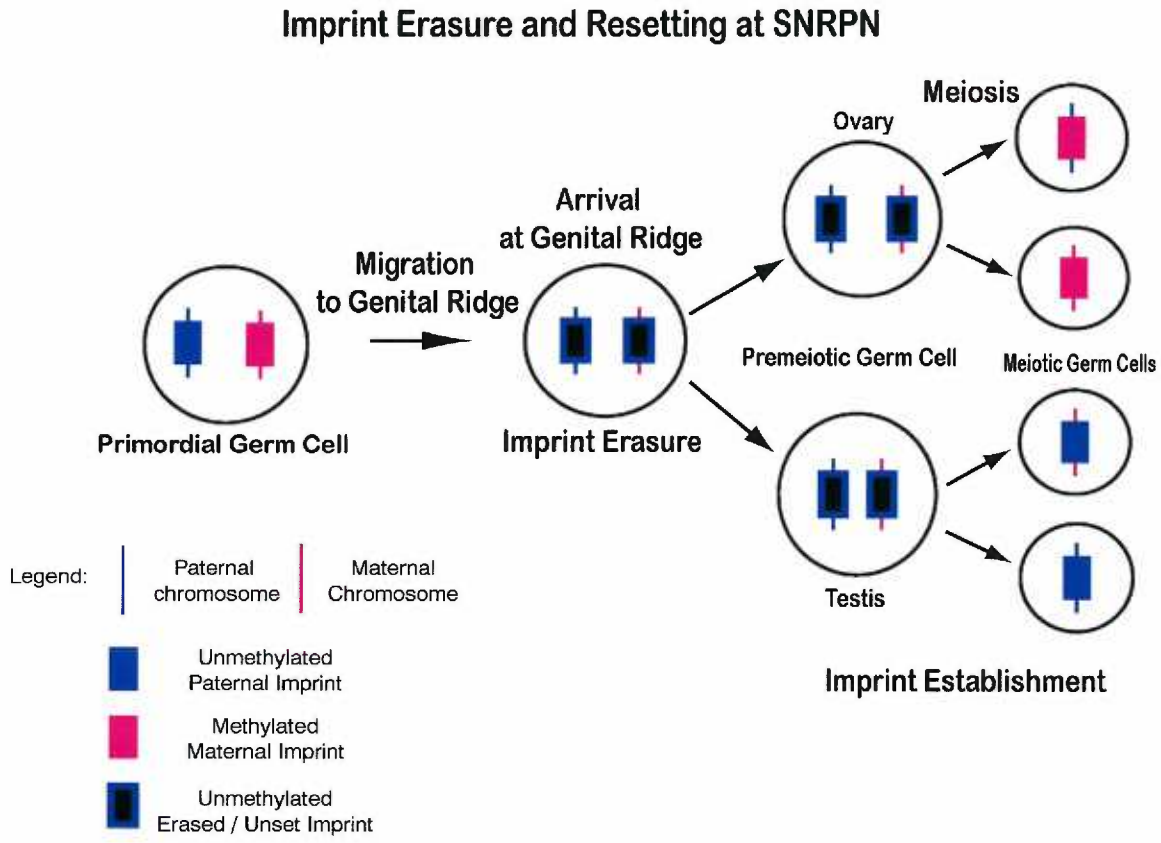
1. Primordial germ cells that have yet to reach the genital ridge will have a methylation pattern identical to the somatic cell pattern. These cells will give rise to tumors in the coccyx and sacrum.

2. Germ cell tumors arising in the gonads will have patterns dependent on the timing of resetting the imprint during gametogenesis and the sex of the patient. For males, this will result in only an unmethylated band being present. For the females, if the cell had yet to re-establish imprinting, the tumor will have only an unmethylated fragment present. If the cell had entered meiosis, then the tumor will have only the methylated

fragment present.

3. Germ cell tumors of the midline that are progressively more anterior to the gonads represent cells that mis-migrated and failed to reset the parental-specific imprint at SNRPN. These tumors will have only an unmethylated fragment.

Figure 1.1: Model of Imprint Resetting at SNRPN



The results and conclusions from these four specific aims are presented in the following papers. "Chromosome abnormalities of 81 pediatric germ cell tumors: sex-, age-, site-, and histopathology -related differences. A Children's Cancer Group Study" reports the cytogenetic characterization of 81 pediatric germ cell tumors. The targetted examination of 1p loss and chromosome 12 abnormalities through FISH is detailed in "Chromosome 1 and chromosome 12 abnormalities in pediatric germ cell tumors detected by interphase FISH." The last two papers present the data from specific aim 4 as it was examined in cultured material from

pediatric GCTs ("Establishing the timing of imprint switching in the germ line using *SNRPN* methylation patterns of pediatric germ cell tumors: implications for loss of imprinting in cancer") and frozen tissue from both adult and pediatric tumors ("*SNRPN* methylation patterns in frozen tissues of adult and pediatric germ cell tumors: determining the timing of tumor formation"). Following the papers, a final summary and discussion of the findings of this project is presented.

Chromosome Abnormalities of 81 Pediatric Germ Cell Tumors: Sex-, Age-, Site- and Histopathology-Related Differences A Children's Cancer Group Study

Kimberly J. Bussey[1,2], Helen J. Lawce[1,2], Susan B. Olson[1,2], Diane C. Arthur[1,3], Dagmar K. Kalousek[1,4], Mark Krailo[1,5], Roger Giller[1,6], Stephen Heifetz[1,7‡], Rick Womer[1,8], and R. Ellen Magenis[1,2].*

[1]Children's Cancer Group, Arcadia, CA; [2]Dept. of Molecular and Medical Genetics, Oregon Health Sciences University, Portland; [3]National Cancer Institute, Bethesda, MD; [4]University of British Columbia, Vancouver; [5]Dept. of Preventive Medicine, University of Southern California, San Diego; [6]Dept. of Hematology-Oncology, Denver Children's Hospital, CO; [7]Dept. of Pathology, Indiana University Medical Center, Indianapolis, IN; [8]Division of Oncology, Children's Hospital of Philadelphia, PA

*Corresponding Author:

R. Ellen Magenis, MD,

Cytogenetic Research Laboratory,

Oregon Health Sciences University,

707 SW Gaines Rd., CDRC 2276,

Portland, OR 97201

Ph: (503) 494-2794 FAX: (503) 494-6104

‡Deceased

Abbreviated Title: Cytogenetic Analysis of 81 Pediatric GCTs

ABSTRACT

The chromosomes of 81 pediatric germ cell tumors (GCT) were analyzed as part of two clinical treatment trials, INT-0098 and INT-0097, conducted by the Children's Cancer Group. The analysis of chromosome results showed differences with respect to sex, age, tumor location, and histology. Sixteen of 17 benign teratomas of infants and children less than 4 years old from gonadal and extragonadal locations were chromosomally normal. Twenty-three malignant GCTs from gonadal and extragonadal locations of the same age group were endodermal sinus tumors and varied in their karyotypic findings. The most common abnormalities were gains of 1q and 3. Of eight benign ovarian teratomas from older girls, five with normal G-band karyotypes were determined to be homozygous for Q-band heteromorphisms, suggesting a meiosis II error. Among the 12 malignant ovarian GCTs from older girls, the common abnormalities were loss of 1p/gain of 1q, +3, +8, +14, and +21. Four of eight extragonadal tumors from older boys demonstrated +21; one had +X. Five of the eight had constitutional chromosome abnormalities including one trisomy 21 and three with Klinefelter syndrome. The testicular GCTs of adolescents had abnormalities resembling those found in adult testicular GCT including near-triploidy, loss of chromosomes 11, 13, and 18 and gain of chromosomes 7, 8, the X chromosome and an isochromosome 12p. The gain of an isochromosome 12p was only frequent in the tumors from adolescent boys. Deletion of 1p/gain of 1q and +3 were the most common abnormalities among the malignant tumors from both sexes.

INTRODUCTION

Germ cell tumors (GCT) are rare tumors in childhood, comprising about 3% of all pediatric malignancies (Young and Miller 1975). The designation GCT reflects the presumed cell of origin, the primordial germ cell, for a heterogeneous group of tumors of multiple histologic types. These tumors occur in both gonadal and extragonadal sites, with approximately 2/3 of pediatric GCT being extragonadal (Harms and Janig 1986). The frequency of specific histologic types, anatomical location, clinical behavior and course of

GCT differs between children and adults (Harms and Janig 1986).

Because of the scarcity of pediatric GCT, few large scale studies of the chromosomal changes in these tumors have been reported. Most of the literature consists of single case reports (Hecht et al. 1984; Ihara et al. 1984; Oosterhuis et al. 1988; Shen et al. 1990; Speleman et al. 1990; Speleman et al. 1992; Albrecht et al. 1993; de Bruin et al. 1994; Stock et al. 1996). The most common findings among these reports were abnormalities of chromosome 1, most often a deletion of distal 1p. An isochromosome 12p, a common finding in adult testicular tumors, was seen in tumors from both gonadal and extragonadal sites in adolescent males (Shen et al. 1990; Albrecht et al. 1993; de Bruin et al. 1994; Stock et al. 1996). This chromosome has also been seen in female tumors, although with considerably lower frequency (Speleman et al. 1990). Two studies have been published which examined the chromosomes in a series of pediatric germ cell tumors. Hoffner et al. (1994) found simple numerical chromosome abnormalities in four ovarian tumors; three of these had malignant components such as endodermal sinus tumor (EST) and embryonal carcinoma (EC). They also studied the origin of the tumors by Q-banding and molecular methods and concluded that, unlike ovarian teratomas, which may arise from a meiosis I or II error, endoreduplication of a haploid ovum, or mitotic division of a premeiotic germ cell (Linder et al. 1975; Parrington et al. 1984; Surti et al. 1990), testicular and extragonadal GCTs in children arise from the mitotic division of a germ cell or somatic cell. A study of nine pediatric ESTs revealed six tumors with chromosome abnormalities (Perlman et al. 1994). Five of the six tumors had structural abnormalities of chromosome 1. Five of the six tumors also showed deletions of 6q, and three showed structural rearrangements of 3p. No instances of i(12p) or abnormalities of chromosome 12 were found.

Three studies, using interphase fluorescence in situ hybridization (FISH) of paraffin-embedded pediatric GCTs, especially EST, with probes for the centromeric regions of chromosomes 1, 8, and 12, and the X chromosome, as well as the midi-satellite at 1p36, identified numerical abnormalities most commonly involving chromosome 12 and loss of

1p36 (Stock et al. 1994; Jenderny et al. 1995; Jenderny et al. 1996). These probes were chosen because of the involvement of these chromosomes in adult testicular GCT. The studies permitted assessment of the localization of abnormal signals versus specific histology. No chromosome abnormalities were identified in teratomatous portions of the tumors studied. Others have reported a similar loss of 1p36 by FISH in the direct harvests of pediatric ESTs (Perlman et al. 1996).

In this study, 81 pediatric GCT were collected as part of treatment trials for advanced high risk malignant germ cell tumors (INT-0098) and localized malignant and immature germ cell tumors (INT-0097) and karyotyped by cytogeneticists at institutions affiliated with the Children's Cancer Group (CCG). The purpose was to evaluate the chromosomes for abnormalities and to elucidate any patterns that might exist.

MATERIALS AND METHODS

Patients and Tumor Pathology

A total of 91 tumors were collected and processed for cytogenetics. Ten of these (6 from female patients and 4 from male patients) were excluded from analysis for one of the following reasons: culture time of more than 10 days, patient age, or pathologic diagnosis inconsistent with GCT. The remaining 81 tumors were from 51 female and 30 male patients. The patients ranged in age from newborn to 18 years, with average ages of 5.3 years and 6.4 years for girls and boys, respectively. The tumors included the following histologic types: mature teratoma (MT), immature teratoma (IT), malignant teratoma (TM), mixed germ cell tumor (MXD), endodermal sinus tumor (EST), embryonal carcinoma (EC), germinoma (GE), and choriocarcinoma (CC).

Sample Collection

Fresh tumor tissue samples were obtained for cytogenetic analysis and were transported as soon as possible in medium or sterile saline to the participating cytogenetic laboratories. Five milliliters of anticoagulated blood was to be submitted to either the institutional labora-

tory or with the tissue sample for constitutional studies.

Tissue Culture and Harvesting

Whole blood was cultured to yield high resolution chromosomes for constitutional studies (Yunis and Chandler 1977; Francke and Oliver 1978). Harvests of tumor cells from the transport medium, using 3 hour Colcemid (50 μg/ ml) after overnight growth, methotrexate synchronization with 3 hour Colcemid exposure, or overnight Colcemid (30 μg/ ml) exposure, were done when possible. Tumor tissue was mechanically and enzymatically dissociated and placed in short-term culture. Several sets of conditions were used for each tumor, sample size permitting. These included using three media: 1) Chang C (Irvine Scientific), 2) RPMI 1640 supplemented with 15% FBS, 2% L-glutamine, 1% insulin-transferrin-selenium, and 1% gentamicin, or 3) a 1:1 mixture of the two. In addition, cells were cultured on either plain or fibronectin-coated T-25 flasks. Six to ten cultures were initiated from each sample. Samples were grown at 37°C in a 5% CO_2 atmosphere. Cultures at 50%-70% confluency were harvested at intervals up to 10 days to maximize the detection of abnormal cells while minimizing the percentage of normal fibroblasts. Metaphase cells were collected following either 3 hr. Colcemid exposure or overnight Colcemid. Some cultures were synchronized with methotrexate prior to the 3 hr. Colcemid exposure. After Colcemid, cells were trypsinized, collected, and incubated in 0.075 M KCl (prewarmed at 37°C) for 10-15 minutes. Cells were then fixed twice with 3:1 methanol:acetic acid and used to make slides immediately or stored at -20°C.

Cytogenetic Analysis

Tumor cell suspensions were dropped on wet slides, air dried, and baked for 20 minutes at 90°C-95°C. Slides were GTW-banded. When possible, at least twenty cells were examined by microscope and photographed for each case. Heteromorphism analysis using quinacrine staining to determine a possible meiotic origin was performed on teratomas from female patients (Overton et al. 1976; Olson et al. 1986). Ten to twenty cells were examined

by microscope and serial photographic prints. FISH to confirm chromosomal rearrangements was done on five patients according to manufacturer's protocols with the following probes: Gibco BRL WCP 12 (#8368 SA), Gibco BRL WCP 12 Orange (#8369 SB), Imagenetics (Vysis, Downers Grove, IL) Chromosome 12 a-satellite (#132012), Oncor Coatasome 11 (P5202-DG.2, Oncor, Gaithersberg, MD), and Oncor Coatasome 12 (P5213-DG.2).

Slides from lymphocyte suspensions were made as described above. Twenty cells were examined by microscope and/or photograph for each case.

Chromosome Segment Representation Profile (CSRP) Analysis

CSRP analysis was done as described by Thompson et al. (1993), with a modification. This type of analysis takes into account the presence of numerical abnormalities, as well as structurally altered homologs in order to assess the recurrent gains and losses for each chromosome. The result is a pictorial representation of the net gain or loss of whole chromosomes or chromosome segments. In this method, multiple copies of a given numerical or structural abnormality are counted individually, and a line for each copy is placed next to the appropriate region on an ideogram (ISCN 1995), with losses to the left and gains to the right. CSRPs of tumors with known location and histology were all described relative to both a diploid progenitor as well as the tumor ploidy. Only those changes that were consistent between the two methods are reported as common abnormalities. We have indicated multiple copies of a given abnormality from each case by the width of the line to eliminate bias from polyploid tumors.

RESULTS

Tumor Distribution By Sex

There were 51 tumor samples from female patients and 30 tumors from male patients. Previous epidemiologic data (Harms and Janig 1986) suggests the higher numbers in tumors from girls as compared to boys may be attributed to a higher proportion of extragonadal

tumors, particularly sacrococcygeal, in girls less than 2 years old, as well as ovarian tumors in the 10- to 14-year old range (see Table 1). In both sexes, almost half [47.1% (24/51) girls, 43.3% (13/30) boys] of all tumors occurred in children under 2 years old. Among the girls, the 24 tumors from this age group were extragonadal, with 15 being sacrococcygeal. The 12 tumors from the boys less than 2 years old were more evenly distributed, with five testicular tumors, four sacrococcygeal, and three tumors from other extragonadal sites (Table 2.1). There were no mediastinal or thoracic tumors among the girls. Additionally, there was a striking demarcation of anatomic location with respect to age among the girls, such that, among the girls 10 years old and older, there were no extragonadal tumors. This is in contrast to the tumors from the boys, in which such a demarcation did not exist. Finally, there was a difference in the incidence of specific histologic types. There were more female patients with tumors of an MT or IT histologic type compared to the tumors from male patients [47.1% (24/51) of girls versus 23.2% (7/30) of boys], and more male patients had a tumor composed of pure EST compared to the tumors of female patients [36.7% (11/30) versus 5.8% (3/51)].

Constitutional Chromosome Studies

Twenty-four patients had constitutional chromosome studies. Six were identified as having constitutional abnormalities. Of these six, five were male [three 47,XXY, one 47,XY,+21, and one 46,X,inv(Y)(p11.1q11.2)], and one was female, 47,XXX. All of the males with constitutional abnormalities had mediastinally or thoracically located tumors. The tumor from the girl with 47,XXX was ovarian.

Cytogenetic Analysis of Tumors

The extragonadal teratomas (11 mature and 5 immature) of infants and young children (4 years old or younger), both male and female, were cytogenetically normal with the exception of one retroperitoneal grade 3 IT from a girl which showed +18 (see Tables 2.2 and 2.3 for detailed results). The one confirmed mature teratoma of the infantile testis was

also chromosomally normal. Malignant GCTs developing at extragonadal locations and in the gonads of infants and young children were exclusively EST. Of these 20 tumors (9 from boys and 11 from girls), 13 were cytogenetically normal. The only recurrent abnormalities among the 10 tumors showing aberrations were rearrangements of chromosome 1 involving a gain of 1q and +3 (Figs. 2.1 and 2.2). No tumor in this category demonstrated an i(12p). There were no detectable differences among the tumors from boys versus those from girls; however, there were only two malignant tumors from boys in this age range with abnormalities. The one malignant GCT from an extragonadal location in a girl 7 years old, case 32, shares common abnormalities seen in the malignant GCTs from infants and young children, namely the gain of 1q and +3.

There were eight ovarian teratomas (four mature and four immature). Five were cytogenetically normal by G-banding. Five (including four of the previous five) were identified by chromosome heteromorphism analysis as having identical centromeric heteromorphisms between homologous chromosomes (Fig. 2.3), one of which was from a 6-year-old, and were presumed to be of parthenogenetic origin. One of these parthenogenetic tumors, a mature teratoma, also had a +8. The other two ovarian teratomas had single numerical gains of 3 and 7.

Twelve malignant ovarian tumors were of mixed histologic types; 10 had a teratoma component (seven IT and three MT) in addition to the malignant tissue. One was chromosomally normal. One was determined to be parthenogenetic. Seven of the twelve were near-diploid with simple numerical changes and had a teratoma component. The most common abnormalities seen when the results from all of the malignant ovarian GCTs were combined, regardless of the presence of teratoma, were loss of 1p/gain of 1q, +3, +8, +14, and +21 (Fig. 2.4). Since the component (malignant or teratoma) provided/cultured for analysis is unknown, the peri-diploid results may represent the mature or immature teratoma portion. Previous flow cytometry studies suggest that the cells with malignant histology in ovarian GCTs are usually polyploid in the triploid to tetraploid range. The only recurrent abnormal-

ity among the seven malignant ovarian GCTs with a teratoma constituent was +14. Among the three malignant ovarian tumors in which it is probable the malignant tissue was cultured, loss of 1p/gain of 1q, +3, +8, +14, and +21 were shared. Only one malignant ovarian tumor had an i(12p) as part of a complex karyotype.

There were nine extragonadal GCTs from boys ages 5 to 18. Two were chromosomally normal. Two had abnormalities that were consistent with the constitutional karyotype (+X and +21) and one had a balanced translocation. The remaining four tumors had primarily simple numerical gains involving chromosome 21 (3 cases) (Fig. 2.5). Gain of the X chromosome was constitutional in two of these four cases and acquired in one case, leading to a pattern of simultaneous gain of both 21 and X.

Of the five malignant testicular GCTs from patients ages 9 to 18, four showed abnormalities of chromosome 12; two had an i(12p). Other abnormalities commonly found in adult testicular GCTs were seen (Fig. 2.6), including near-triploidy with loss of chromosomes 11 (four cases), 13 (three cases), and 18 (two cases) and gain of chromosomes 7 (two cases) and 8 (one case) and the X chromosome (one case).

DISCUSSION

The behavior of GCT in children as compared to that of GCT in adults has suggested that they may be pathogenetically distinct entities. Differences in the number of extragonadal tumors, the prevalence of specific histologic types, and the clinical behavior have been reported (Harms and Janig 1986). Other studies have also identified differences between the ploidy levels in pediatric GCT and that of adults. Adult testicular GCT is usually hypotetraploid for seminomas and hypertriploid for nonseminomas (de Jong et al. 1990), whereas pediatric GCT are usually diploid or near-tetraploid (de Jong et al. 1990; Silver et al. 1994). Our study supports this distinction between GCT in children and adults and suggests that patient sex and age impact the histologic types and cytogenetic abnormali-

ties seen in pediatric GCT. Furthermore, the chromosomal abnormalities in relationship to sex, age, site, and histopathology permit the classification of pediatric GCTs into six groups: 1) gonadal and extragonadal teratomas of infants and young children, 2) malignant GCTs of infants and young children, 3) ovarian teratomas, 4) malignant ovarian GCTs, 5) testicular tumors of pubertal and post-pubertal males, and 6) extragonadal tumors of older and adolescent boys.

Tumors from group 1, the gonadal and extragonadal teratomas, both mature and immature, of children 4 years-old and younger, were cytogenetically normal with the exception of one case (case 16). The malignant GCTs of children in this age range, which comprise group 2, were EST, either alone or in conjunction with a teratoma portion. The most common abnormalities of this group of tumors were gain of 1q and +3. Perlman et al. (1994) observed similar findings in their study of nine infantile pure EST. We did not observe the chromosome arm 6q deletions they described in their patient series as a common abnormality in our series. This may reflect a difference in the abnormalities associated with pure EST versus those associated with tumors of mixed histologic types, having both EST and MT or IT. Of our three cases of pure EST, two had deletions of 6q. Perhaps the deletion of 6q is a change related to the development of a purely malignant phenotype in these tumors. The one pure EST from an extragonadal location in a 7 year-old girl (case 32) had not only a gain of 1q and +del(3)(p21), but a deletion of 6q as well, suggesting this tumor may be more like the malignant tumors from infants and children than the malignant ovarian tumors, which are more common for girls of that age.

While the cytogenetic data do not support a separation of the tumors of infants and young children, either benign or malignant, into categories based on sex, our data recommends caution should be taken combining the results of tumors in this age range from both sexes. First, the number of tumors we received from girls in this age range was higher than that of the boys (1.9 times). This can be attributed to the greater number of sacroccocygeal teratomas among girls as compared to boys. While this may be a result of sampling bias, it is

similar to other studies (Harms and Janig 1986). Secondly, there was a difference in the frequency of benign versus malignant histologic types between the sexes, with 11 of 27 girls having malignant tumors versus 9 of 14 boys. The frequency of particular histologic types also differed between the sexes, with boys more commonly having a pure EST (eight of nine malignant tumors), while only two of the eleven malignant tumors from girls were pure EST. Finally, it must be kept in mind that the numbers of tumors with abnormalities detected by G-band analysis from either sex in this age range was small, and any differences between the sexes in abnormalities might not be apparent.

The cytogenetic results from group 3, the benign ovarian teratomas, resembled those found in adults. The prevalence of tumors arising from a meiosis II error or endoreduplication of a haploid ovum, as determined by centromeric heteromorphism analysis, was 62.5%, similar to the 65% reported by Surti et al. (1990). The three benign teratomas with abnormalities had simple numeric gains, again similar to the adult counterparts. Benign ovarian teratomas from adults show gains of 3 and 8 to be common (Ihara et al. 1984; Jenkyn and McCartney 1987; Yang-Feng et al. 1988; Dahl et al. 1990; Surti et al. 1990). Two of the three cases with chromosome abnormalities in our series exhibited either +3 or +8. These results imply that the benign ovarian teratomas seen in the pediatric age range could be related to those found in adult women.

The most common abnormalities identified in malignant ovarian GCTs, group 4, were loss of 1p/gain of 1q, +3, +8, +14, and +21. Note that the one malignant ovarian GCT of a 2 year old had a gain of 8 and 14 as the only abnormalities, similar to the aberrations seen in the malignant ovarian GCTs of older girls.

The component received and cultured for analysis in mixed tumors is unknown. It could be argued that based on flow cytometry studies (Kommoss et al. 1990; Silver et al. 1994), the near-diploid results obtained on tumors with an IT portion represent the IT and not the malignant elements. However, after eliminating the tumors with near-diploid results from analysis, the list of common abnormalities for malignant ovarian GCTs in our data does not

change. Furthermore, the one abnormality the near-diploid malignant ovarian GCTs share is +14. The results reported in the literature suggest that the common gains of whole chromosomes we observed can be found in malignant ovarian GCT with teratomous elements (Hoffner et al. 1994; Rodriguez et al. 1995). This raises two possibilities. First, the abnormalities found in near-diploid malignant ovarian GCTs with teratoma may represent the initial chromosome changes associated with the acquisition of a malignant phenotype. Along these lines, cytogenetic and flow cytometric studies of IT show an association of aneuploidy with increasing grade, such that only the ITs of grade 3 were aneuploid (Ihara et al. 1984; Silver et al. 1994). The other possibility is that the common whole chromosome gains seen in malignant ovarian GCTs are not related to the malignant phenotype. Gains of chromosomes 3, 8, and 21 have been observed in mature ovarian teratomas without reported evidence of malignant degeneration (Dahl et al. 1990; Surti et al. 1990; Speleman et al. 1992). Careful studies in which the teratoma is cultured and analyzed separately from the malignant elements in mixed ovarian GCTs are required to address this question.

Gains of 21 and X were consistent findings in group 5, the extragonadal tumors of older boys. They occurred as both acquired and constitutional changes. The association of constitutional chromosome abnormalities, such as Klinefelter syndrome, trisomy 21, and others leading to dysgenetic gonads, with a higher risk of GCT, particularly mediastinal tumors, has been reported (Scully 1970; Schellhas 1974; Sogge et al. 1979; Curry et al. 1981; Turner et al. 1981; Mann et al. 1983; Muller et al. 1985; Nichols et al. 1987; Hasle et al. 1992; Derenoncourt et al. 1995; Dieckmann et al. 1997; Satge et al. 1998). Our data shows a frequency of 47,XXY of 10% among the male patients, compared with a 0.16% frequency in the general male population (Robinson and de la Chapelle 1997).

The testicular tumors of pubertal and post-pubertal boys comprise group 6. The karyotypic abnormalities were virtually identical to those seen in adult testicular tumors, especially in the presence of the i(12p) (de Jong et al. 1990; Chaganti et al. 1993). In adults, testicular GCT is primarily near-triploid, with seminomas being hyper-triploid to tetraploid

and nonseminomas being in the hypo-triploid range (de Jong et al. 1990). Cytogenetic analysis has revealed that 80% of adult testicular germ cell tumors contain an i(12p) (Rodriguez et al. 1993; Heim and Mitelman 1995). In the i(12p) negative tumors, rearrangements of the short arm of 12, particularly involving 12p11.2-12, have been identified (Rodriguez et al. 1993; Suijkerbuijk et al. 1993; Korn et al. 1996; Mostert et al. 1996). In the studies of male germ cell tumors that demonstrate an i(12p) or amplified 12p sequences, those tumors from children were from patients no younger than 15 years old (Suijkerbuijk et al. 1993; Korn et al. 1996). Note that the one male non-testicular tumor in our series demonstrating an i(12p) (case 70) is likely to be in the pubertal category.

In this context, we agree with others (Perlman et al. 1994; Stock et al. 1996) that i(12p) is restricted to tumors from pubertal or post-pubertal boys and absent in pre-pubertal GCT. This suggests that the pathogenesis of these tumors is influenced by the physiologic changes brought about during puberty. Therefore, the tumors from pre-pubertal males are likely a separate etiological entity from the pubertal and adult counterparts. This is supported by the fact that carcinoma *in situ*, a common finding in adult testicular tumors, is absent in the overwhelming majority of pre-pubertal tumors; only one case has been reported (Hu et al. 1992).

The loss of 1p/gain of 1q and gain of chromosome 3 are the only two changes which are common among the malignant GCTs of infants and young children, and the gonadal GCTs of older children and adults. The imbalance between sequences on 1p and 1q, particularly del(1p36), in pediatric GCT has been reported by several groups (Perlman et al. 1994; Stock et al. 1994; Perlman et al. 1996; Stock et al. 1996). All of the tumors in our series that displayed this abnormality had a malignant germ cell component. Loss of 1p has been associated with poor outcome or aggressive behavior in several tumor types, including neuroblastoma, ductal breast cancer, colorectal carcinoma, melanoma, Wilms tumor, and endometrial cancers (Grundy et al. 1994; Ambros et al. 1995; Gerdes et al. 1995; Walker et al. 1995; Arlt et al. 1996; Farabegoli et al. 1996). Stock et al. (1994) found deletions of 1p

only in the malignant components of mixed GCT. Introduction of intact short arms of chromosome 1 into a neuroblastoma cell line with a 1p deletion resulted in a proliferation stop of the cells (Bader et al. 1991). Tanaka et al. (1993) reported that microcell fusion hybrids of a colon carcinoma cell line, COKFu, containing 1p34-p36 demonstrated a suppressed transformed phenotype. This suggests that tumor suppressor genes located on distal 1p are likely to be involved with the ability of tissues to undergo terminal differentiation. As such, the deletions of 1p/gains of 1q in pediatric GCT may be indicative of malignancy and might serve as a prognostic indicator of tumor behavior.

ACKNOWLEDGMENTS

We are grateful to Dr. Theodore Pysher and all of the participating CCG institutions that submitted tumor material for this study. We thank Mike Brown for his help in preparing figures for the manuscript and David Adler, Department of Pathology, University of Washington, Seattle, for the use of Ideogram Albums provided on the World Wide Web.

TABLE 2.1 Distribution of Tumors by Age, Site, and Histology

Age		Site			
		Gonadal	Sacrococcygeal	Mediastinal/ thoracic	Other Extragonadal
<2 years	Female		6 MT 3 IT 3 MT/EST 1 IT/EST 2 EST		3 MT 1 IT 3 IT/EST 1 U
	Male	1 MT 3 EST 1 U	2 MT 2 EST		1 MT 1 IT 1 EST
2-4 years	Female	1 IT 1 IT/EST	1 MT /EST		1 MT
	Male	1 EST	2 EST		
5-9 years	Female	1 MT 1 IT/EST 2 MXD			1 EST 1 U
	Male	1 MXD		1 MT 1 MXD	1 MT
10-14 years	Female	3 MT 3 IT2 MT/EST 3 IT/EST 2 MXD 1 U			
	Male		1 TM	1 EST 1 MXD	1 GE
>15 years	Female	1 IT 1 IT/EST 1 MXD			
	Male	4 MXD		1 EST 1 MT/EST	

Abbreviations: MT, mature teratoma; IT, immature teratoma; TM, malignant teratoma; EST, endodermal sinus tumor; GE, germinoma; MXD, mixed tumor; U, unknown histology.

TABLE 2.2: Tumor Data for 51 Female Patients

Case	Age[a]	Site Of Tumor[b]	Tumor Histology[c]	Clinical Stage	Cytogenetic Results[d]
1	1 d	Extragonadal, roof of mouth	MT		46,XX[20]
2	1 d	Sacrococcygeal	MT		46,XX[21]
3*	1 d	Sacrococcygeal	IT, grade 2	I	46,XX[20]
4	2 d	Sacrococcygeal	MT		46,XX[26]
5	3 d	Extragonadal, pelvis	MT		46,XX[22]
6	4 d	Sacrococcygeal	IT		46,XX[19]
7	4 d	Sacrococcygeal	MT		46,XX[20]
8*+	5d	Sacrococcygeal	IT, grade 3/EST	I	46,XX[24]
9	6 d	Sacrococcygeal	MT		46,XX[20]
10	7 d	Sacrococcygeal	MT		46,XX[21]
11‡	10 d	Sacrococcygeal	IT		46,XX[16]
12*	17 d	Extragonadal, head/neck	IT, grade 3/EST	I	47,XX,+i(1)(q10)[20]/46,XX[1]
13	6 wk	Unknown	IT		46,XX[19]
14	55 d	Sacrococcygeal	MT		46,XX[18]
15	2 m	Extragonadal, midline CNS	MT		46,XX[22]
16*	2 m	Extragonadal, retroperitoneal	IT, grade 3	II	47,XX,+18[20]
17*	4 m	Extragonadal, unknown	IT, grade 2/EST	IV	46,XX,der(15)t(1;15)(q21;p11.2)[9]/46,idem, der(22)t(1;22)(q12;p13)[8]/46,XX[3]
18*	7 m	Extragonadal, unknown	IT, grade 2/EST	II	47,XX,+14[1]/46,XX[19]
19*	7 m	Sacrococcygeal	EST	IV	46,XX[20]
20	9 m	Extragonadal, buttocks	U		46,XX[20]
21*	1 y	Sacrococcygeal	MT/EST	II	46,XX[20]
22*	1 y	Sacrococcygeal	MT/EST	IV	46,XX[20]
23	1.4 y	Sacrococcygeal	EST		90<4n>,XX,del(X)(p22)x2,-4,-5,-9,-17,-17,-19, -20,+der(?)t(?;1)?;q12)x2,+mar1,+mar2, +mar3[15]/46,XX[5]
24*	1.4y	Sacrococcygeal	MT /EST		46,XX[20]
25*	2 y	Ovary	IT, grade 1		46,XX,inv dup(1)(p36.3p21)[1]/46,XX[19]
26*	2 y	Ovary	IT, grade 3/ EST	IV	49,XX,+8,+14,+14[20]
27*	2 y	Sacrococcygeal	MT /EST	IV	46,XX[18]
28	2 y	Extragonadal, neck	MT		46,XX[20]
29	6 y	Ovary	MT		(23,X)x2[20]
30*	6 y	Ovary	IT, grade 1/ EST/EC	II	76<3n>,XX,del(X)(q22q26),add(1)(p36),+3,-4, +7,+del(7)(q22q33),+dir dup(8)(q1?1q24), +psu dic(8)t(1:8)(p3?2;q24),+add(12)(p11),-13,+14, +17,-19,+20,+mar [4]/47,XXXc[16]
31*	7 y	Ovary	IT/EST	I	47,XX,+2[5]/47,XX,+3[6]/48,XX,+2,+3[5]/49,XX, +2,+3,+14[4]

32	7 y	Extragonadal, pelvis	EST		44-46,XX,i(1)(q10),add(2)(q37),+2,+del(3)(p21), -4,del(5)(q11.2q13),add(6)(q?21),add(15)(p11), add(18)(p?11),i(19)(q10),-20,?add(22)(q13), +mar[cp16]/46,XX[10]
33	8 y	Extragonadal, abdominal	U		46,XX[16]
34*	8 y	Ovary	MT/EST/EC/GE/CC	I	115<5n>,XXXXX,-1,+3,+3,-4,del(4)(q27q35),-5,+6, +6,+6,+6,-7,+8,+9,-10,-11,+i(12)(p10)x2,-13,+14, -15,-15,-16,-18,-18,-19,-19,+21,+21[cp8]/46,XX[2]
35*	10 y	Ovary	IT, grade 2		47,XX,+3[20]
36	10 y	Ovary	MT		47,XX,+7[20]
37*	10 y	Ovary	IT, grade 3/EST	I	(23,X)x2[23]
38*	10 y	Ovary	IT, grade 3/EST	III	47,XX,+14[12]/46,XX[10]
39	10 y	Ovary	IT		(23,X)x2[31]
40	11 y	Ovary	MT		(23,X)x2[17]
41*	11 y	Ovary	IT, grade 3 /EST	I	47,XX,+14[20]
42*	11 y	Ovary	MT/EST/GE	I	46,XX,+12,-13[20]
43*	12 y	Ovary	GE/EST	III	63<3n>,XX,-X,-1,-3,-4,dup(4)(q31q35),-5, del(6)(q2?1q2?3),+add(9)(q11),-11,-13,-16, der(16)t(1;16)(q21;q13),-18,+21,-22,+mar1, +mar2[5]/60<3n>,XX,-X, add(1)(p36),del(1)(p3?2p36),-3,-4,dup(4)(q31q35), -5,add(9)(q11),-11,-13,-16,der(16)t(1;16)(q21q13), -18,-21,-22,+mar1,+mar2[5]
44	13 y	Ovary			46,XX[54]
45*	13 y	Ovary	MT/EST	III	47,XX,+18[13]/48,XX,t(9;22)(p13;q13),+18,+20[4]/ 46,XX[3]
46€	14 y	Ovary	MT		(23,X)x2,+8[15]
47*	14 y	Ovary	MT /EST	II	47,XX,+17[2]/48,XX,+X,+17[1]/46,XX[24]
48*	14 y	Ovary	IT, grade 3	I	46,XX[20]
49*	15 y	Ovary	IT, grade 3	II	(23,X)x2[21]
50*	16 y	Ovary	IT, grade 3/EST	I	46,XX[23]
51*	16 y	Ovary	EST/EC	I	46,XX[47]

[a]d, day; wk, week; m, month; y, year

[b]Extragonadal refers to any extragonadal site, usually along the midline, other than sacrococcygeal.

[c]MT=mature teratoma; IT=immature teratoma; TM= malignant teratoma; EST=endodermal sinus tumor; EC=embryonal carcinoma; GE=germinoma; CC=choriocarcinoma; MXD=mixed malignant germ cell tumor (tumor has two or more histologies indicative of malignancy); U=Unknown.

[d]All cytogenetic analyses were done by Helen Lawce or Mike Brown except for cases 9, 29, and 46 (KB did Q-band analysis).

*Case reviewed by S. Heifetz, MD.

[†]Received immature teratoma portion only.

[‡]Received non-tumor component.

€Tumor from a Pediatric Oncology Group (POG) affiliated institution.

TABLE 2.3: Tumor Data for 30 Male Patients

Case	Age[a]	Site Of Tumor[b]	Tumor Histology[c]	Clinical Stage	Cytogenetic Results[d]
52	2 d	Sacrococcygeal	MT		46,XY[23]
53*	3 d	Extragonadal, head/neck	IT, grade 3	I	46,XY[20]
54	1 m	Sacrococcygeal	MT		46,XY[17]
55	2 m	Extragonadal, abdominal	IT, grade 2		46,XY[21]
56	2 m	Unknown	U		46,XY[19]
57	4 m	Sacrococcygeal	EST		46,XY[18]
58	5 m	Testis	MT		46,XY[20]
59	6 m	Testis	U		46,XY[18]
60*	11 m	Testis	EST	I	46,XY[20]
61*	1 y	Sacrococcygeal	MT/EST	IV	46,XY[20]
62	1 y	Testis	EST		46,XY[20]
63*	1 y	Testis	EST	I	46,XY[20]
64*	1.5 y	Extragonadal, pelvis	EST	IV	46,XY[7]
65	2 y	Sacrococcygeal	EST	IV	46,XY[23]
66*	2.7 y	Sacrococcygeal	EST	III	48-51,XY,add(1)(p36),+add(2)(q31),+3,+3, add(6)(q2?7),add(10)(q26),add(15)(p11),+mar1, +mar2[7]/ 46,XY[13]
67*	3 y	Testis	EST	I	74<3n>,X,der(X)t(X;17)(q28;q21),-Y,-1, t(1;9)(q21q22),+dic(1;2)(p11;q11.2),+3,-4, -5,i(6)(p10),+del(6)(q13q25),+7,add(8)(q24), +11,+14,add(15)(q22),der(16)t(1;16)(q21;p11), add(18)(q23),+21,+mar1,+mar2[11]
68	5 y	Mediastinal	MT		46,XY[17]
69*	6 y	Mediastinal	MT/GE/EST	I	48,XXYc,+21[12]/47,XXYc[8]
70	9 y	Extragonadal, pineal	U		49,XY,+X,+i(12)(p10),+21[19]
71	9 y	Testis, metastasis to lung	EC/TM		Unanalyzable abnormal [2]/?99,XXYY,-1,-1,-2,-2,-3,-3,-5,-11,-12,-12,-13,-14,-14,-16,-16,+17, +18,+18,+3mar1,+16mar,inc [1]/46,XY[12]
72*	10 y	Mediastinal	MT /EST /EC/CC	II	48,XXYc,+21[9]/49,XXYc,+17,+21[9]/47,XXYc[1]/46,XYc[1]
73	12 y	Sacrococcygeal	TM		46,XY,t(4;19)(q35;q13?.3)[9]/46,XY[13]
74	13 y	Extragonadal, pineal	GE		46,XY[17]
75*	13 y	Extragonadal, thoracic	MT/EST	III	46,X,inv(Y)(p11.2q11.2)c,-13,+21[5]/ 92,idemx2[11]/92,idemx2,del(4)(q22q31)[10]
76*	15 y	Mediastinal	EST	III	47,XY,+21c[17]
77	16 y	Testis	MT/EC/CC/EST		62<3n>,XXY,-1,-4,-5,+psu dic(8)t(1;8)(1pter-1q44;8cen-qter),-9,-9,-10,-11, der(12)t(9;12)(q11;p13),+i(12)(p10),-13, der(15)t(15;21)(q11;p11),add(18)(p11), -22[5]/63,idem,+der(15)t(15;22)(p11;q11)[11]

78	17 y	Testis	EC/CC		68-81<3n>,XXY,+X,+Y,+7, -11,+12, +i(12)(p10)x2,der(13;21)(q10;q10), +der(13;21)(q10;q10),add(18)(p11), +mar1, +mar2 [cp19]/ 46,XY [1]
79	17 y	Testis	EC/SE/TM		60-68<3n>,XXY,-4,-9,-10,-11, +der(12)?t(12;12)(pter-q13:p11.2-pter)x2,-13,-16,-18,-22 [cp7]/60-68,idem, del(6)(q?13q31)[cp8]/46,XY[4]
80*	18 y	Extragonadal, thoracic	MT/EST	III	47,XXYc
81*	18 y	Testis	MT/EC/CC	III	66-70<3n>,XXY,der(1)t(1;5)(p11;p11), +der(1)t(1;11) (p?36;q11),-4, -6,+7,+7, dic(9;11)(p?22;q?23),+dic(9;11) (p?22;q?23),-10,-11,-11, add(12)(q?13),dic(12;16)(p11;q?24), +i(12)(p10),+14,-16,-17,-18 der(19)t(?17;19) (q11;q?13.1),+21,inc [cp20]

[a]d, day; wk, week; m, month; y, year

[b]Extragonadal refers to any extragonadal site, usually along the midline, besides sacrococcygeal.

[c]MT=mature teratoma; IT=immature teratoma; TM=malignant teratoma; EST=endodermal sinus tumor; EC=embryonal carcinoma; GE=germinoma; CC=choriocarcinoma; MXD=mixed malignant germ cell tumor (tumor has two or more histologies indicative of malignancy); U=Unknown.

[d]Cytogenetic analyses done by Helen Lawce and Mike Brown

* Case reviewed by S. Heifetz, MD.

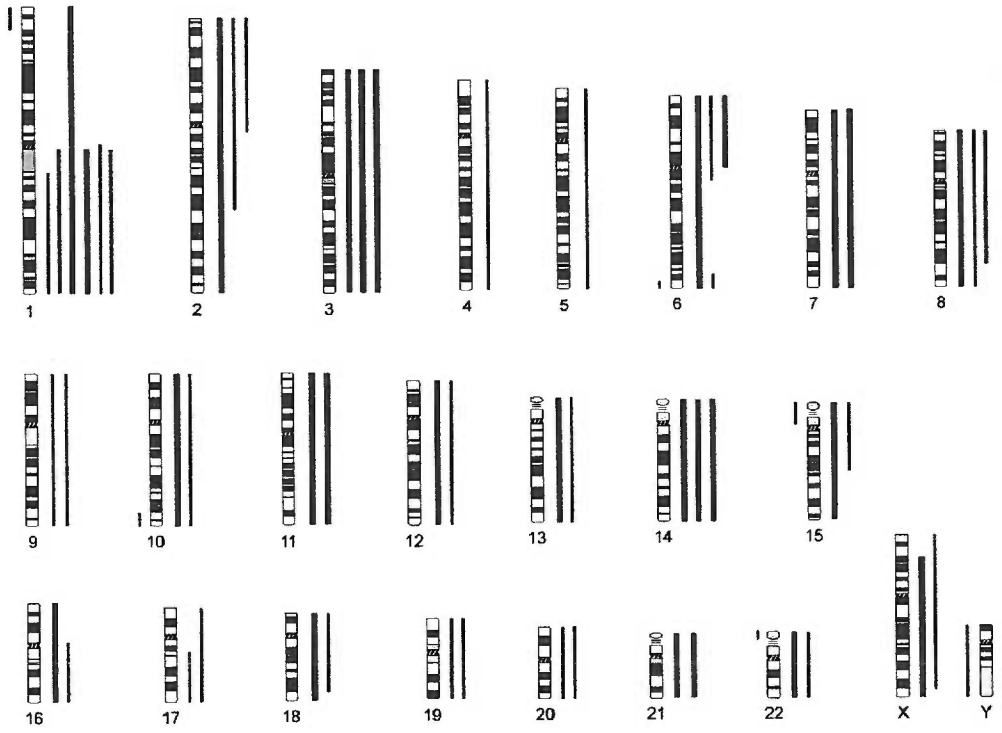


Figure 2.1. Chromosome segment representation profile (CSRP) for malignant GCTs from children 4 years old and younger. All abnormalities were counted relative to diploid. Losses are on the left and gains on the right of each ideogram (ISCN 1995). Each line represents a single event from a single case, with the copy number of the abnormality indicated by the width of the line.

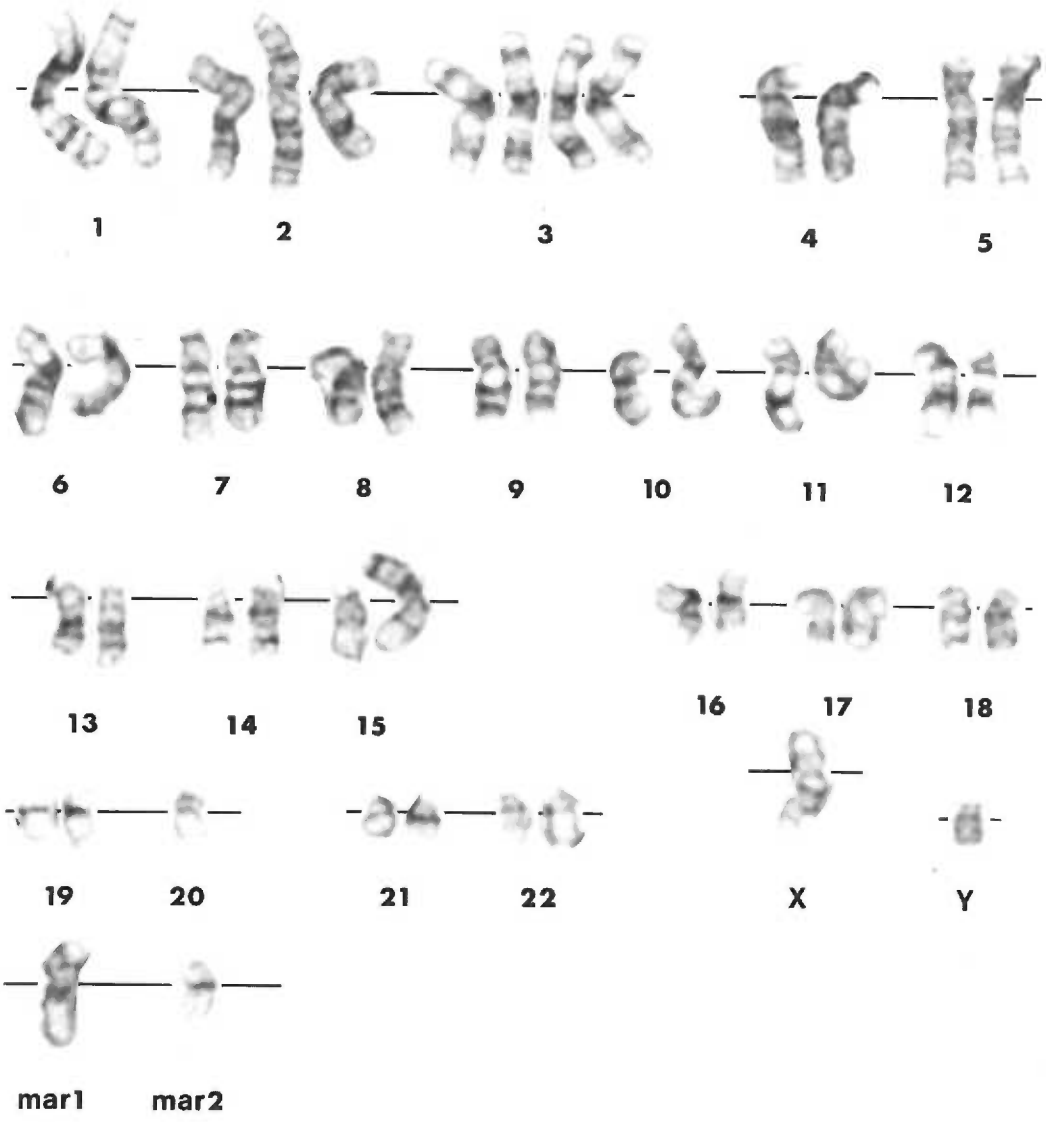


Figure 2.2. A representative G-banded karyotype from a malignant infantile GCT demonstrating the common changes of loss of 1p, +3, and loss of 6q. Additional changes are +add(2)(q31), add(10)(q26),add(15)(p11),+mar1,+mar2.

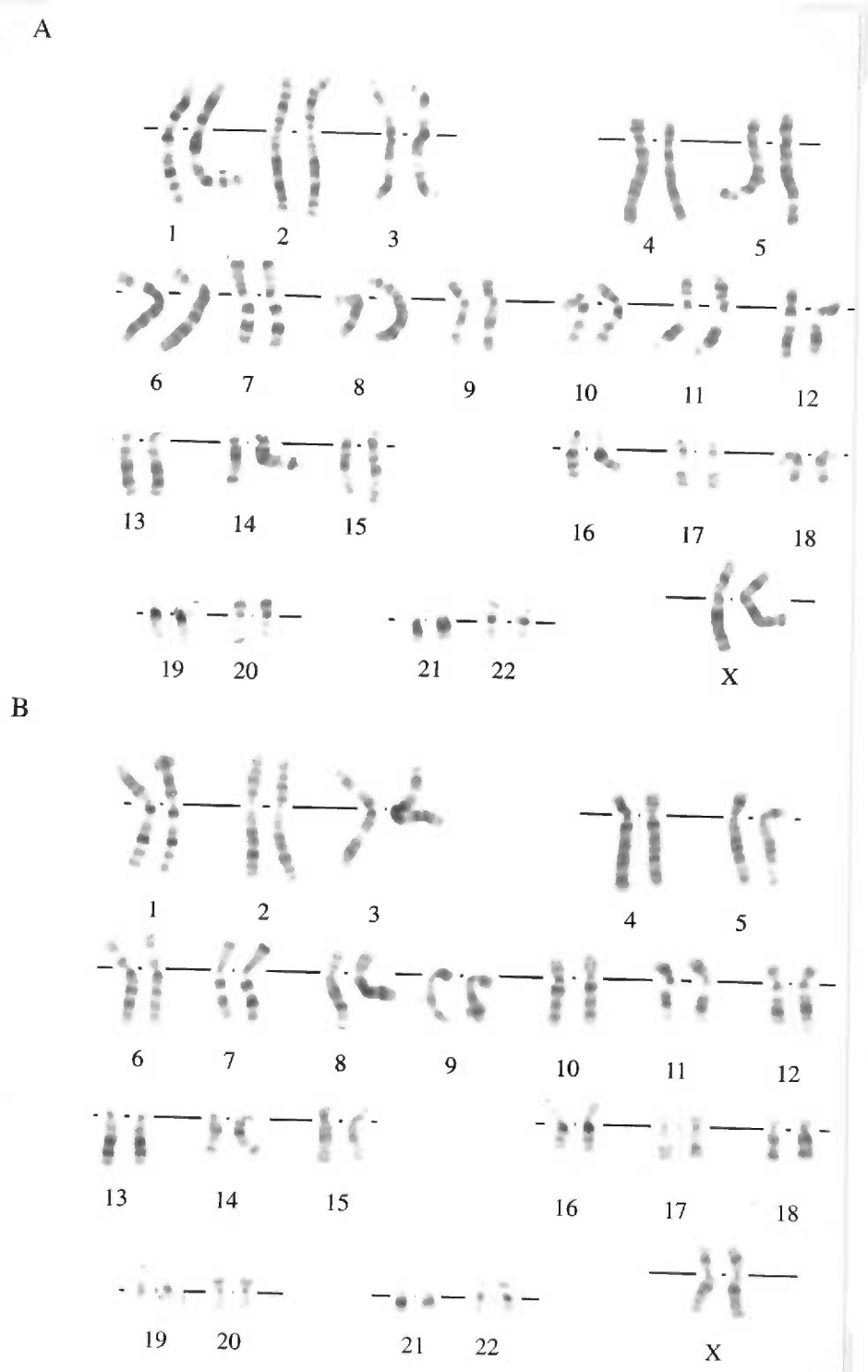


Figure 2.3. Representative G-banded karyotypes from A) the constitutional study and B) a 46,XX,parthenogenetic tumor from the same individual. There are differences between homologs in the heterochromatic regions of 1, 9, 16, and the short arm/stalk/satellite regions of the acrocentrics in A), whereas in B), these regions show no difference between the homologs, indicating a meiosis II error. For example, in A) the left homolog of chromosome 1 has a small block of heterochromatin in the q arm, while the homolog on the right has a large block of heterochromatin. In B) both homologs have the smaller heterochromatic region.

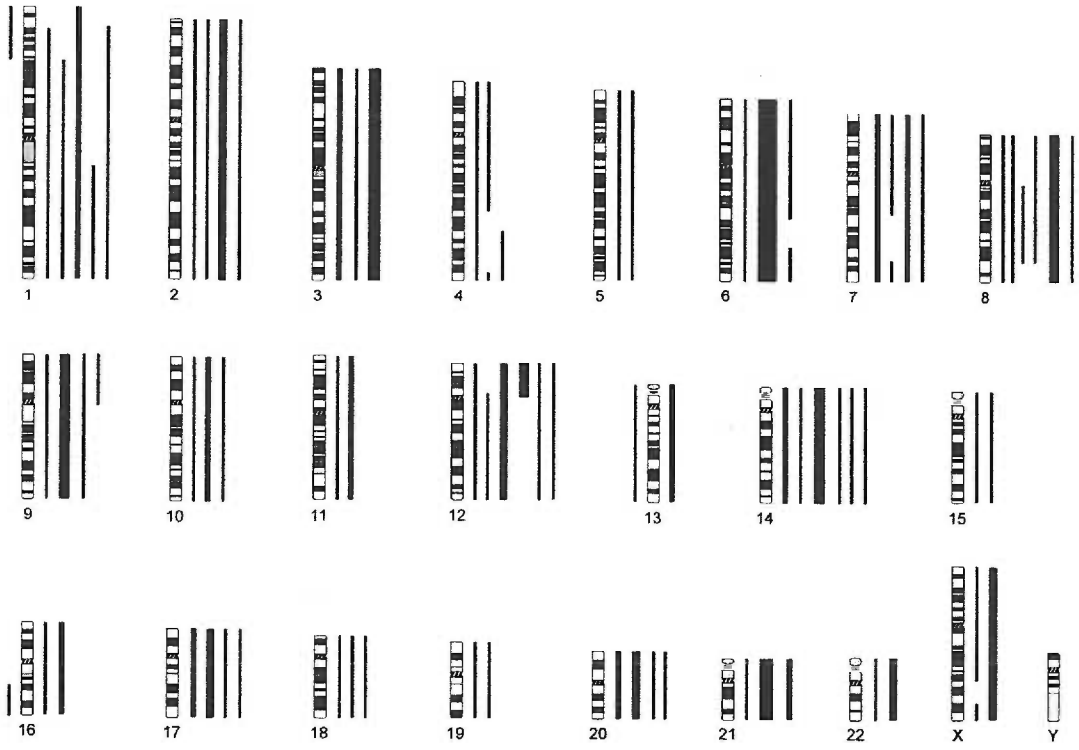


Figure 2.4. Chromosome segment representation profile (CSRP) for malignant ovarian GCTs of older girls. All abnormalities were counted relative to diploid. Losses are on the left and gains on the right of each ideogram (ISCN 1995). Each line represents a single event from a single case, with the copy number of the abnormality indicated by the width of the line.

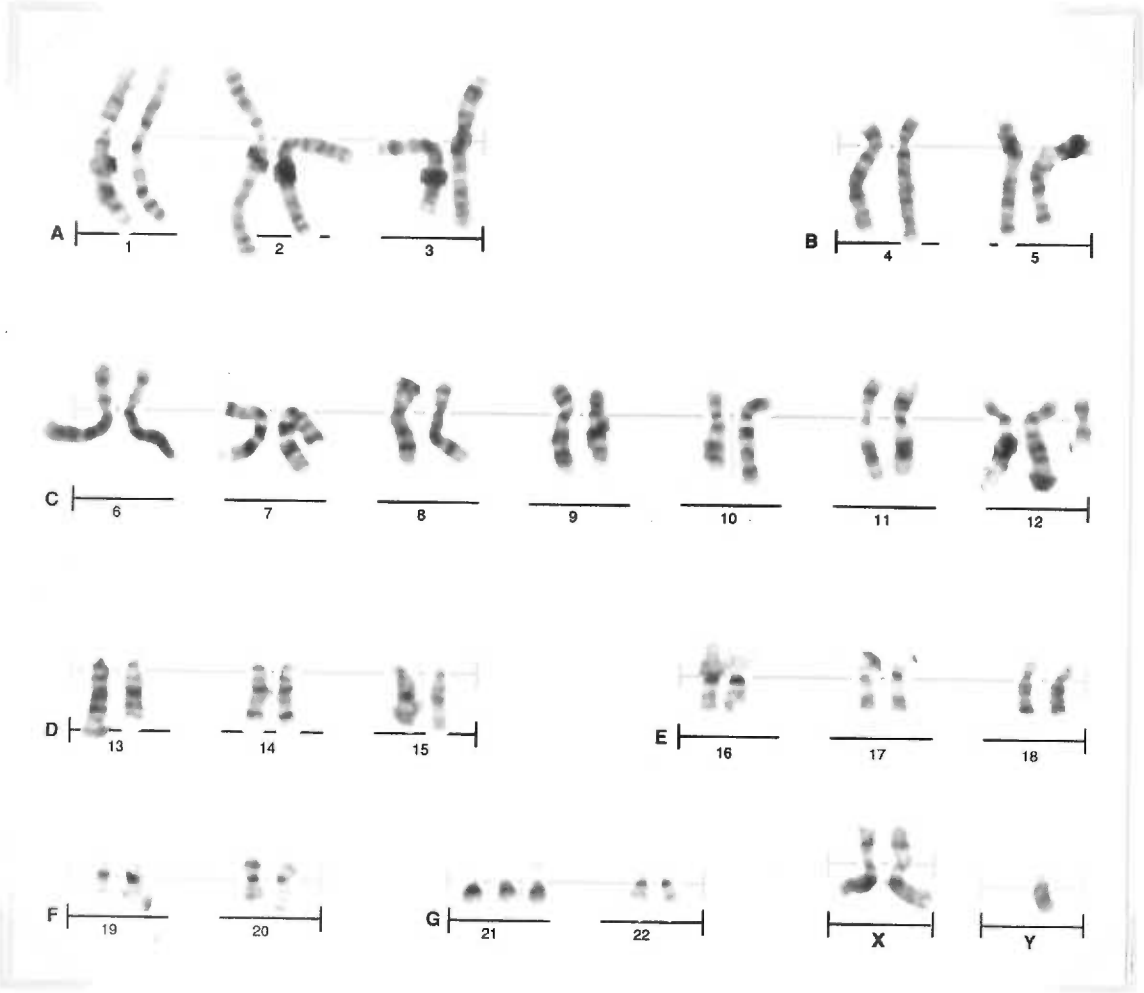


Figure 2.5. Karyotype of pineal teratoma from case 70, showing 49,XY,+X,+i(12)(p10),+21.

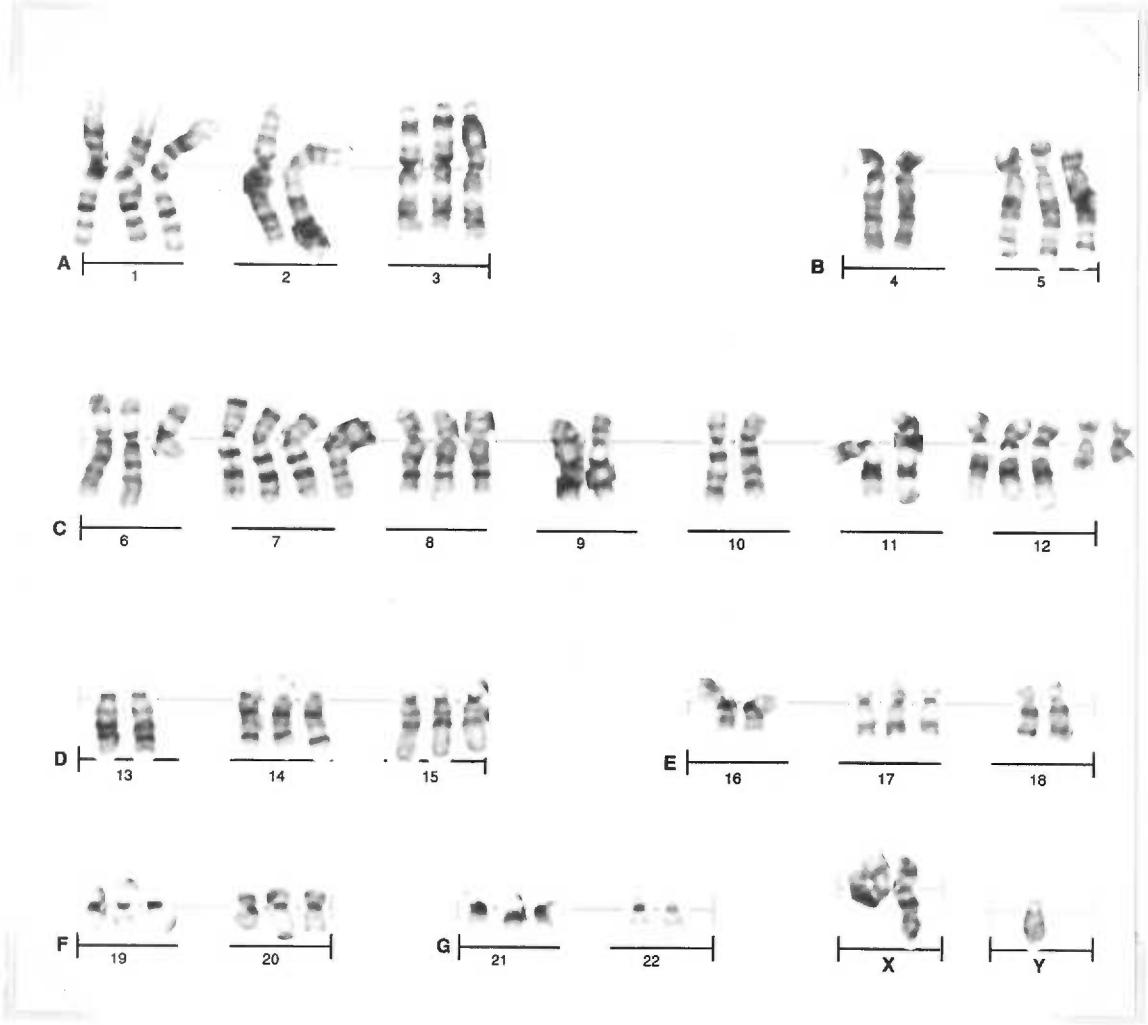


Figure 2.6. Representative abnormal G-banded karyotype from the malignant testicular tumor of a post-pubertal male.

Chromosome 1 and chromosome 12 abmormalities in pediatric germ cell tumors detected by interphase FISH

Kimberly J. Bussey[1,4], Helen J. Lawce[1], Eleanor Himoe[1,4], Xiao Ou Shu[2,4], Ron F. Suijkerbuijk[3] Susan B. Olson[1], and R. Ellen Magenis[1,4*]

[1]Department of Molecular and Medical Genetics, Oregon Health Sciences University, Portland OR

[2]Department of Pediatrics, University of South Carolina, Columbia SC

[3]Department of Human Genetics, University Hospital, Nijmegen, The Netherlands

[4]Children's Cancer Group, Arcadia CA

*Corresponding Author:

R. Ellen Magenis, MD,

Cytogenetic Research Laboratory

Oregon Health Sciences University

3183 SW Sam Jackson Park Rd., MP 350

Portland, OR 97201

Ph: (503) 494-2794 FAX: (503) 494-6104

ABSTRACT

Chromosome studies of germ cell tumors (GCTs) in children have shown differences in abnormalities dependent on age, sex, tumor location, and histology. Cytogenetic studies of particularly one type of pediatric GCT, endodermal sinus tumor, have identified rearrangements of chromosome 1, with loss of 1p36. Amplification of 12p material, especially as an i(12p), is a common finding in adult testicular GCT but uncommon in childhood GCT. However the incidence of chromosome 12 abnormalities leading to a gain of 12p material remains undefined in pediatric GCT. Fifty-three pediatric GCTs were analyzed for loss of 1p36 by GTW banding and dual-color interphase FISH with probes for D1Z5 (chromosome 1 alpha satellite) and D1Z2 (1p36.3). Twelve tumors were identified with a loss of 1p36. No deletion was detected in tumors with non-malignant histology, such that there is a significant association of 1p loss with malignancy in pediatric GCTs ($p=0.00115$). Eighteen tumors from male patients were analyzed for 12p amplification using Pα12H8, which detects the chromosome 12 alpha-satellite, and YAC5 (12p11.2-12.1). Five tumors had amplification of 12p, consistent with G-band results. Combined analysis of our data with the literature revealed a significant correlation of 12p amplification with patient age ($p=0.000196$). Amplification of 12p was seen only in 1 of 35 tumors from females patients. Five female GCTs had numerical abnormalities of chromosome 12, and 2 tumors showed complete lack of 12p. This spectrum of abnormalities differs from that seen in the male tumors, providing further evidence for different etiologies of GCTs between the sexes.

INTRODUCTION

Germ cell tumors (GCTs) are rare in the pediatric patient population. Although heterogeneous, with both benign and malignant presentations in the gonads as well as extragonadally, these tumors share a common presumed cell of origin, the primordial germ cell. Recent studies have identified distinct subgroups with differences in the cytogenetic abnormalities present. Benign teratomas from children four years old or younger are cytogenetically normal (Hoffner et al. 1994; Bussey et al. 1999). Malignant GCTs from children

four years old or younger are predominantly endodermal sinus tumors (ESTs) and are characterized by gains of 1q and abnormalities of chromosomes 3 and 6 (Perlman et al. 1994; Bussey et al. 1999). The benign ovarian teratomas of older girls (greater than 4 years old) resemble those of adults, both showing evidence of parthenogenesis and simple numeric gains (Ihara et al. 1984; Ohama et al. 1985; Surti et al. 1990; Bussey et al. 1999). The malignant ovarian GCTs of older girls are characterized by loss of 1p/gain of 1q and whole chromosome gains of chromosomes 3, 8, 14, and 21 (Speleman et al. 1990; Speleman et al. 1992; Hoffner et al. 1994; Bussey et al. 1999). The extragonadal tumors of older boys, which present primarily in the mediastinum or pineal region, are usually near-diploid or near-triploid with gains of the X chromosome and/or chromosome 21 (Shen et al. 1990; Albrecht et al. 1993; Casalone et al. 1994; de Bruin et al. 1994; Sole et al. 1994; Bhattacharjee et al. 1997; Lemos et al. 1998; Bussey et al. 1999). The testicular tumors of adolescents resemble those of adults with near-triploidy that includes relative loss of chromosomes 11, 13, and 18 and relative gain of the X chromosome, chromosomes 7 and 8, as well as an i(12p) (de Jong et al. 1990; Chaganti and Houldsworth 1998; Bussey et al. 1999).

Two abnormalities have been reported to be common among more than one subgroup listed above. These are loss of 1p and amplification of 12p. Previous investigations with FISH attempted to address the incidence of loss of 1p and numerical copy number of chromosome 12 in the pediatric population. Stock et al. (1994) found that additional copies of chromosome 12 were the most frequently observed numerical abnormality, but not i(12p) as indicated by no change in chromosome 12 centromeric signal size. Additionally, loss of 1p36 was restricted to sites of malignant histology, even in tumors with both a benign and a malignant component. Jenderny et al. (1995) had similar findings in 12 tumors. A study of ESTs from children less than 4 years old demonstrated loss of 1p36 in eight of ten tumors examined (Perlman et al. 1996).

The purpose of this study was to assess the incidence of loss of 1p36 and the amplification of 12p in a series of pediatric GCTs collected as part of studies conducted

collaboratively with the Children's Cancer Group. All had been previously analyzed by G-banding (Table 3.1 and Bussey et al. 1999). The question was to determine whether loss of 1p was associated with a malignant phenotype in our tumors, as previously reported (Stock et al. 1994; Jenderny et al. 1996). It was also questioned whether the lack of 12p amplification detected by G-band analysis was truly reflective of the tumors studied, particularly among the tumors from pre-pubertal boys, and what the spectrum of chromosome 12 abnormalities in tumors from females was.

METHODS

Sample Collection and Tissue Culture

Fresh tissue from pediatric GCTs was collected as part of Children's Cancer Group (CCG) protocols 8882, 8891, and E-22. Tumor tissue was mechanically and enzymatically dissociated and placed in short-term culture. Several sets of conditions were used for each tumor, sample size permitting. Three media were used: (1) Chang C (Irvine Scientific), (2) RPMI 1640 supplemented with 15% FBS, 2% L-glutamine, 1% insulin-transferrin-selenium, and 1% gentamicin, or (3) a 1:1 mixture of the two. In addition, cells were cultured on either plain or fibronectin-coated T-25 flasks. Six to ten cultures were initiated from each sample. Samples were grown at 37°C in a 5% CO2 atmosphere.

Harvests of tumor cells from the transport medium were done when possible using 3-hour Colcemid (50 μg/ml) after overnight growth, methotrexate synchronization with 3-hour Colcemid exposure, or overnight Colcemid (30 μg/ml) exposure. Cultures at 50% to 70% confluency, initiated as described above, were harvested at intervals up to ten days to maximize the detection of abnormal cells while minimizing the percentage of normal fibroblasts. Metaphase cells were collected following either 3-hour Colcemid exposure or overnight Colcemid. Some cultures were synchronized with methotrexate prior to the 3-hour Colcemid exposure. After Colcemid exposure, cells were trypsinized, collected, and incubated in 0.075 M KCl (pre-warmed at 37°C) for 10 to 15 minutes. Cells were then fixed

twice with 3:1 methanol:acetic acid and used to make slides immediately or stored at -20°C. Tumor cell suspensions were dropped on wet slides and air dried.

Interphase FISH Analysis

For analysis of loss of chromosome 1p36.3, FISH was performed with a biotin-labeled chromosome 1 alpha satellite (Oncor) and either digoxigenin-labeled p1-79 (courtesy of Michael Litt, Ph.D.), which recognizes D1Z2, or $p58^{clk-2}$ (Oncor), which hybridizes to the PITSLRE kinase gene locus (*CDC2L1*). 12p amplification was detected using CY3-labeled pα12H8 (ATCC) and digoxigenin-labeled YAC5. Slides were treated in 2XSSC for 30 minutes at 37°C, dehydrated in an ethanol series at room temperature, and air dried. If necessary, excessive cytoplasm and cellular debris was removed by pepsin digestion for 3 to 13 minutes at 37°C prior to the ethanol series. Slides were then denatured in 70% formamide/ 2XSSC for 5 minutes at 74° to 76°C, followed by dehydration in an ice-cold ethanol series. Probes were denatured separately at 70° to 74°C for 10 minutes. Alternatively, slides and probes were co-denatured at 75°C for 3 minutes. Slides were hybridized overnight at 37°C. The slides were then post-washed in 60% formamide/ 2XSSC (p1-79) or 50% formamide/ 2XSSC ($p58^{clk-2}$ and YAC5) at 43°C for 15 minutes followed by 8 minutes in 2XSSC at 37°C. Probes for chromosome 1 were detected with rhodamine-anti-digoxigenin and FITC-avidin (Oncor) for 15 minutes at 37°C. YAC5 was detected with FITC-anti-digoixgenin (Roche Molecular Biochemicals) for 15 minutes at 37°C. The slides were rinsed three times, 2 minutes each in PN (0.1 M Na2PO4 with 0.1% Nonident-40), and counterstained with DAPI (100 ng/μl). Slides were visualized on a Zeiss Axiophot equipped with a Cytovision system (Applied Imaging). When possible, at least 300 nuclei were scored for the presence of each probe. One of ten normal control tumor samples, harvested at approxiamtely the same time as the patient samples, was probed on every run. The data were used to create a tolerance interval for each combination of signals observed. The interval was chosen at a 95% confidence of having 95% of the data fall within the cut-off values. Only those tumors with signal distributions outside of the tolerance intervals were

considered to demonstrate loss or gain.

RESULTS

Chromosome 12 FISH

Interphase FISH analysis of 12p amplification of 18 GCTs from male patients revealed amplification in three tumors (Table 3.2 and Fig. 3.1). When these data were combined with previous G-band analysis (Table 3.1, Bussey et al. 1999), five tumors had amplification of 12p. One tumor, case 79, lost the chromosomally abnormal clone in culture, and one tumor, case 81, had a marker chromosome containing the chromosome 12 alpha-satellite as well as an i(12p). No amplification of 12p was observed in tumors from patients less than 9 years old. Although our data set is too small to reach significance, by combining our data with that reported in the literature, 12p amplification is significantly associated with patient age, such that it is only found in tumors from boys 9 years old or older, with an odds ratio of 42.37 (one-tailed Fisher's exact, p=0.000196).

The frequency of 12p amplification in female GCTs is not well established. We observed only one tumor of 35 tumors from female patients, a mature teratoma with a normal female karyotype, to have amplification of 12p (case 103, Table 3.3). Aneuploidy for chromosome 12 was observed in five tumors. In two of these tumors (cases 23 and 88), trisomic or tetrasomic cell populations were detected. One tumor (case 24) had a cell population with six copies of chromosome 12. These three tumors had a malignant component. Two tumors had cell populations that were monsomic for chromosome 12. Case 32 was chromosomally normal by G-bands and had two copies of chromosome 1 by FISH, indicating monosomy 12 was not indicative of a haploid cell population. The other tumor, case 91, had a normal female karyotype, but had evidence for a haploid cell population by chromosome 1 FISH as well. Two additional tumors had a cell population with complete loss of 12p. Both tumors were benign from girls less than four years old.

Chromosome 1 FISH

Interphase FISH analysis with probes for the short arm of chromosome 1 and the alpha satellite of chromosome 1 was performed on a total of 53 GCTs from children 18 years old or younger. Twelve tumors demonstrated loss of 1p (see Table 3.4 and Fig. 3.2). Three-quarters of tumors with 1p loss had no detectable loss by G-band analysis. In one case, deletion of 1p was present in metaphases that were unanalyzable by G-bands; the remaining eight cases showed no evidence of 1p deletion in metaphase. Nine of the 12 tumors with loss of 1p had confirmed malignant histology, showing a significant association of 1p loss with malignant phenotype, with an odds ratio of 28.17 (one-tailed, Fisher's exact, p=0.00115). There was no loss of 1p observed in tumors from children less than one year old. Although the numbers are small, there appeared to be a difference between the frequency of 1p loss and patient sex, age, and tumor location. Among the tumors from female patients, two of six malignant tumors from children 4 years old or younger had loss, while four of five malignant tumors from older girls had loss of 1p. There appeared to be no association with location other than what has been previously seen for tumor location and patient age (Bussey et al. 1999). Among the tumors from male patients, one of two malignant tumors from boys 4 years old or younger had loss of 1p. Malignant tumors from the older male patients showed a loss of 1p in three of five gonadal and one of five extragonadal tumors.

DISCUSSION

The role of individual chromosome aberrations in carcinogenesis is a continuing question. We examined two specific chromosome abnormalities by interphase FISH that had previously been reported to be common in GCTs- loss of 1p36 and amplification of 12p. Isochromosome 12p is a hallmark of adult testicular GCTs. It is rarely found, however, in tumors from young boys. Since traditional methods for chromosome studies, such as G-banding, require dividing cells, it has been argued that the lack of 12p amplification observed by G-banding in the GCTs from young boys was artifact resulting from not catching the clone in metaphase. Our data show this not to be the case. In fact, 12p amplification is

significantly associated with age, being restricted to boys 9 years old or older. This suggests that the underlying biology for 12p amplification may be in part due to physiological changes brought about by the onset of puberty. It also argues for different etiologies for GCTs depending on the age of the patient.

Previous work on the presence of i(12p) in tumors from the pediatric female suggested that i(12p) is not a common change; only 3 cases have been reported (Speleman et al. 1990; Speleman et al. 1992; Bussey et al. 1999). Numerical abnormalities of chromosome 12 are more common (Hoffner et al. 1994; Stock et al. 1994; Jenderny et al. 1996; Bussey et al. 1999). Again, our FISH data are in agreement with the literature. We observed only one case of 12p amplification and gain of chromosome 12 in three tumors. The gain of chromosome 12 material was associated with a malignant phenotype. Previous work shows gain of 12 is primarily found in complex karyotypes (Speleman et al. 1990; Speleman et al. 1992; Bussey et al. 1999), suggesting chromosome 12 abnormalities represent a possible secondary event. Among malignant GCTs from adult women, i(12p) appears to be more common and is associated primarily with dysgerminomas (Atkin and Baker 1987; Rodriguez et al. 1995; Baker et al. 1998; van Echten et al. 1998). As with tumors from males, this indicates there may be some age-related selection for 12p amplification in the malignant female GCTs. We did discover a novel finding of what appears to be complete lack of 12p signals in two benign tumors from young girls. Further study is required to determine whether this is a specific abnormality for this age group or an artifact.

Loss of 1p has been reported in several tumor types and is often an indicator of poor prognosis. We found a significant association of 1p loss with malignancy in GCTs, regardless of age or sex of the patient. Our experience also suggests that the incidence of 1p loss in GCTs as assessed by interphase FISH is considerably higher than found by G-banding alone. Previous studies show most malignant GCTs in the pediatric population have loss of 1p, particularly 1p36, when assayed by FISH (Jenderny et al. 1995; Perlman et al. 1996; Stock et al. 1996). Our overall incidence of 1p loss is lower than previously reported studies. This

most likely represents a difference in using short-term cell culture versus paraffin-embedded sections. The literature shows little difference in the incidence of 1p loss between male and female patients or with relationship to age. Our data shows a possible difference in the incidence of 1p loss with respect to patient age and sex, but the data set is too small for any significant comparisons. Additional investigations are needed to further define this relationship and establish its importance.

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Table 3.1: Cytogenetic Analysis of Previously Unreported Tumors

CASE	KARYOTYPE[a]
85	46,XX[13]
90	46,XX[23]
93	48,XX,+7,+8[21]
94	46,XY[24]
100	79-93<2N>,XX,+X,+I(1)(q10),+del(1p),+2,+3,+3, +del(3q),+4,+I(5)(p10),+6,+8,+9,+11,+11,+12,+13,+14, +14,+16,+16,+17,+19,+19,+21,+22,+6-13mar,inc[18]
101	45~51,XY,+X,+1,+3,-5,add(6q),+7,-8, ?add(9p),+10,-10,+14,-15,+17, +1-4mar[cp7]/78~97<2n>,XY,+X,+X,+Y,+1,+1,+2,+2,+3,+3,+3,+3,+4, +4,+5,+add(6q)x2,+7,+7,+7,+8,+9,+9,+10,+10,+11,+11,+12,+13,+13,+14,+14, +add(15p),+15,+16,+16,+17,+17,+18,+18,+19,+19,+19,+20,+20,+21,+22,+22, +4-6mar[cp6]/46,XY[12]
102	63~88<3N>,XXY,+X,+Y,+1,+2,del(3)(q?),+del(3)(q?),-5, +7,+8,+8,-10, +der(11)t(1;11)(q12;q?21), i(17)(q10),+21,+21,+mar1,+mar2,inc[7]/46,XY[15]

[a]Cytogenetic analysis of cases 93 and 100 done by Helen Lawce. Case 94 analyzed by Eleanor Himoe.

Table 3.2: Results of FISH for 12p Amplification in 18 GCTs from Male Patients

CASE	RESULTS[a]
56	2/2 (78%)
59	2/2 (71%)
94	2/2 (67%)
62	2/2 (82%)
101	2/2 (76%)
69	2/2 (68%)
70	3/4 (56%) 2/2 (9%)
71	2/2 (87%)
72	2/2 (75%)
84	2/2 (63%)
99	2/2 (80%)
83	2/2 (63%)
102	2/2 (23%) 4/4 (16%)
77	4/5 (49%)
95	2/2 (86%)
78	2/2 (9%) 5/7 (11%) 6/7+ (29%)
79	2/2 (88%)
81	5/5 (40%) [i(12)(p10) by G-banding]

[a]Results are presented as number of chromosome 12 α-satellite signals per nucleus/ number of YAC5 signals per nucleus, with the percentage of cells in parentheses. Normal control sample will have 42% to 100% of cells in the 2/2 category with the remaining cells distributed over 28 other combinations of signals. Only this category and any other category that had cells outside of the normal range (a tolerance interval at 95% confidence that 95% of the data fall within the interval) are reported.

Table 3.3: Results of FISH for 12p Amplification in 35 GCTs from Female Patients

CASE	RESULTS[A]
1	2/2 (73%)
2	2/2 (71%)
92	2/2 (67%)
5	2/2 (77%)
6	2/2 (69%)
8	2/2 (69%)
10	2/2 (87%)
97	2/2 (82%)
13	2/2 (35%) 2/0 (25%)
14	2/2 (87%)
15	2/2 (70%)
103	2/2 (67%) 2/3 (6%)
19	2/2 (78%)
20	2/2 (64%)
96	2/2 (76%)
23	2/2 (53%) 3/3 (8%) 4/4 (10%)
24	2/2 (63%) 6/6 (8%)
98	2/2 (79%)
100	2/2 (49%)
28	2/2 (59%) 2/0 (15%)
87	2/2 (89%)
90	2/2 (80%)
29	2/2 (85%)
82	2/2 (75%)
31	2/2(71%)
32	2/2 (63%) 1/1 (9%)
36	2/2 (69%)
39	2/2 (67%)
93	2/2 (75%)
85	2/2 (78%)
86	2/2 (65%)
88	2/2 (68%) 4/4 (13%)
46	2/2 (77%)

91	2/2 (50%) 1/1 (16%)
89	2/2 (86%)

[a]Results are presented as number of chromosome 12 α-satellite signals per nucleus/ number of YAC5 signals per nucleus, with the percentage of cells in parentheses. Normal control sample will have 42% to 100% of cells in the 2/2 category with the remaining cells distributed over 28 other combinations of signals. Only this category and any other category that had cells outside of the normal range (a tolerance interval at 95% confidence that 95% of the data fall within the interval) are reported.

Table 3.4: Results of FISH for 1p Loss in 53 Pediatric Germ Cell Tumors

Case	Patient Age[a]	Tumor Location	Histology[b]	Results[c]	
				p1-79	p58[clk-1]
1	1 d	Extragonadal, mouth	MT	2/2 (67%)	ND
2	1 d	Sacrococcygeal	MT	2/2 (72%)	ND
92	2 d	Sacrococcygeal	MT/IT, grade 1, stage I	2/2 (76%)	ND
5	3 d	Extragonadal, pelvis	MT	2/2 (87%)	ND
6	4 d	Sacrococcygeal	IT	2/2 (67%)	ND
8	5 d	Sacrococcygeal	IT, grade 3/EST	2/2 (59%)	2/2 (69%)
10	7 d	Sacrococcygeal	MT	2/2 (74%)	2/2 (76%)
97	11 d	Sacrococcygeal	MT/EST	2/2 (73%)	2/2 (59%)
13	6 wk	Unknown	IT	2/2 (84%)	ND
14	55 d	Sacrococcygeal	MT	2/2 (79%)	ND
56	2 m	Unknown	U	2/2 (38%)	2/2 (54%)
15	2 m	Extragonadal, Midline CNS	MT	2/2 (68%)	ND
103	4 m	Extragonadal, Retroperitoneal	MT	2/2 (82%)	ND
59	6 m	Testis	U	2/2 (76%)	ND
94	7 m	Mediastinal	IT	2/2 (68%)	2/2 (61%)
19	7 m	Sacrococcygeal	EST, stage IV	2/2 (73%)	ND
20	9 m	Extragonadal, buttocks	U	2/2 (63%) 4/4 (7%)	ND
62	1 y	Testis	EST	2/2 (83%)	ND
96	1 y	Unknown	U	2/2 (65%)	ND
98	1.17 y	Extragonadal, Retroperitoneal	MT	2/2 (72%)	ND
24	1.4 y	Sacrococcygeal	MT/EST	2/2 (63%) 3+/2- (24%)	2/2 (50%) 3+/2- (25%)
23	1.4 y	Sacrococcygeal	EST	2/2 (76%)	ND
100	1.5 y	Ovary	EST	4/2 (26%) 2/2 (36%)	4/2 (6.4%) 2/2 (52%)
101	1.75 y	Extragonadal, abdomen	EST	2/2 (52%)	2/2 (57%)
28	2 y	Extragonadal, neck	MT	2/2 (67%)	ND
87	2 y	Ovary	IT, grade 2/3	2/2 (71%)	ND
90	5 y	Extragonadal, abdomen	TM	2/2 (60%) 3/2 (14%)	4/2 (11%) 3/2 (14%) 2/2 (41%)
69	6 y	Mediastinal	MT/GE/EST	2/2 (57%)	ND
82	6 y	Ovary	IT, grade 1	2/2 (69%)	ND
29	6 y	Ovary	MT	2/2 (78%)	ND
32	7 y	Extragonadal, pelvis	EST	2/2 (65%)	ND
31	7 y	Ovary	MT/EC/EST	2/2 (81%)	ND
70	9 y	Extragonadal, pineal	U	2/2 (54%) 3/2 (22%)	2/2 (67%) 3/2 (15%)

71	9 y	Testis, lung metastasis	EC/TM	2/2 (63%)	ND
72	10 y	Mediastinal	MT/EST/EC/CC	2/2 (78%)	ND
39	10 y	Ovary	IT	2/2 (80%)	ND
36	10 y	Ovary	MT	2/2 (65%)	2/2 (61%)
93	11 y	Extragonadal, pelvis	U	2/2 (67%)	2/2(70%)
84	11 y	Extragonadal, pineal	GE	2/2 (68%)	ND
86	12 y	Ovary	U	2/2 (17%) 4/3 (17%)	2/2 (15%) 4/3 (23%)
85	12 y	Ovary	GE	2/2 (32%) 4/4 (8%) 5/4 (5%)	2/2 (34%) 4/4 (10%) 5/4 (4%)
99	13 y	Extragonadal, brain	U	2/2 (65%)	ND
88	13 y	Ovary	GE	4/3 (25%) 3/3 (15%) 2/2 (27%)	4/3 (10%) 3/3 (5%) 2/2 (25%)
83	14 y	Extragonadal, thalamus	GE	2/2 (72%)	ND
46	14 y	Ovary	MT	2/2 (74%)	ND
102	15 y	Extragonadal, pineal	GE	4/4 (28%) 4/3 (14%) 3/3 (17%) 2/2 (11%)	4/4 (11%) 4/3 (9%) 4/2 (8%) 3/3 (6%) 2/2 (29%)
91	16 y	Ovary	MT	2/2 (58%) 1/1 (13%) 4/4 (7%)	ND
77	16 y	Testis	MT/EC/CC/EST	3/3 (62%) 3/2 (10%) 4/3 (7%)	3/3 (42%) 3/2 (12%) 4/3 (8%)
95	16 y	Testis	U	2/2 (38%) 2/0 (13%)	2/2 (59%) 3/2 (16%)
89	17 y	Gonad	GE	2/2 (63%)	2/2 (60%)
78	17 y	Testis	EC/CC	3/3 (27%) 2/2 (25%)	ND
79	17 y	Testis	EC/SE/TM	2/2 (74%) 3/2 (13%)	2/2 (56%) 3/2 (19%)
81	18 y	Testis	MT/EC/CC	4/2 (31%) 4/1 (10%) 4/0 (9%)	4/2 (15%) 4/1 (10%) 4/0 (8%)

[a]d, day; wk, week; m, month; y, year

[b] MT=mature teratoma; IT=immature teratoma; TM= malignant teratoma; EST=endodermal sinus tumor; EC=embryonal carcinoma; GE=germinoma; CC=choriocarcinoma; MXD=mixed malignant germ cell tumor (tumor has two or more histologies indicative of malignancy); U=Unknown.

[c]Results are presented as number of chromosome 1 α-satellite signals per nucleus/ number of 1p36.3 probe signals per nucleus, with the percentage of cells in parentheses. A normal control sample will have 48% to 92% of cells in the 2/2 category with the remaining cells distributed over 28 other combinations of signals. Only this category and any other category that had cells outside of the normal range (a tolerance interval at 95% confidence that 95% of the data fall within the interval) are reported.

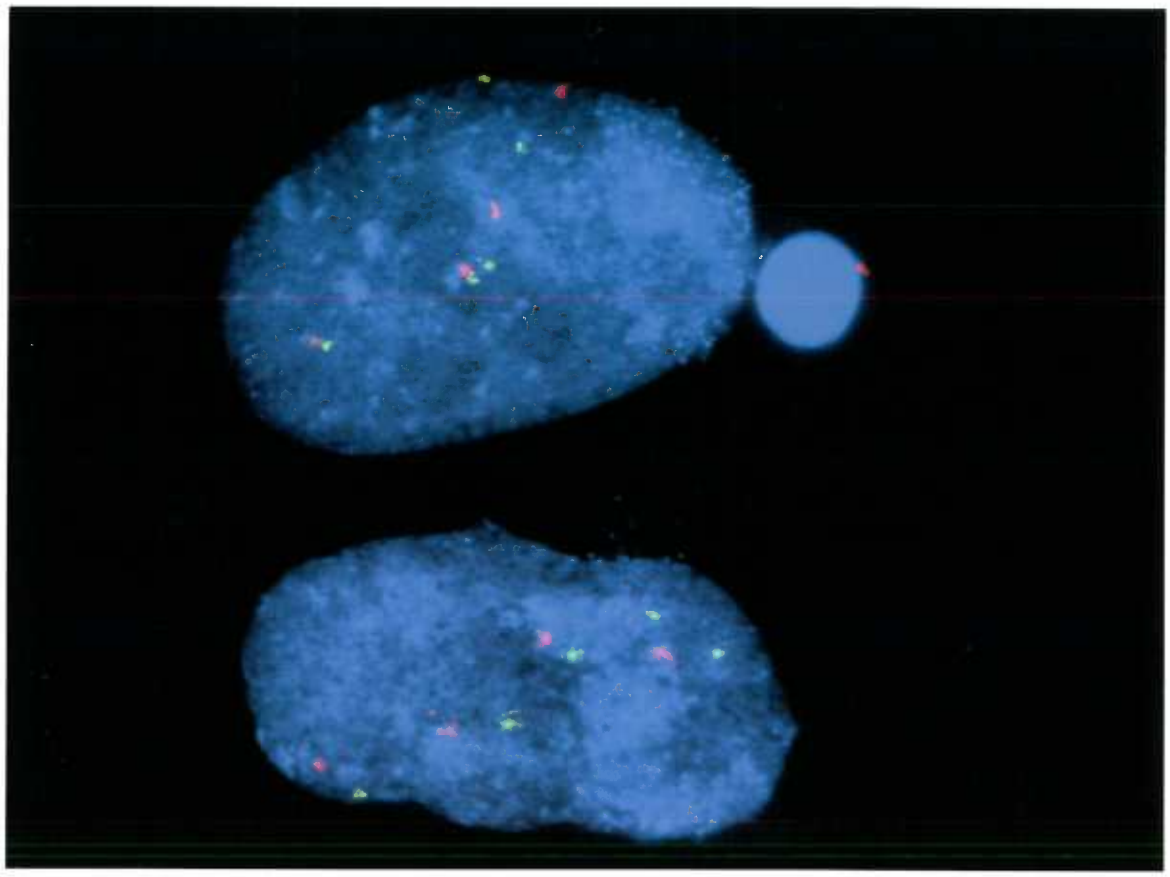

Figure 3.1. Interphase FISH for 12p amplification. There are more YAC5 (green) signals detecting 12p11.2-12.1 compared to the number of 12 alpha-satellite signals (red).

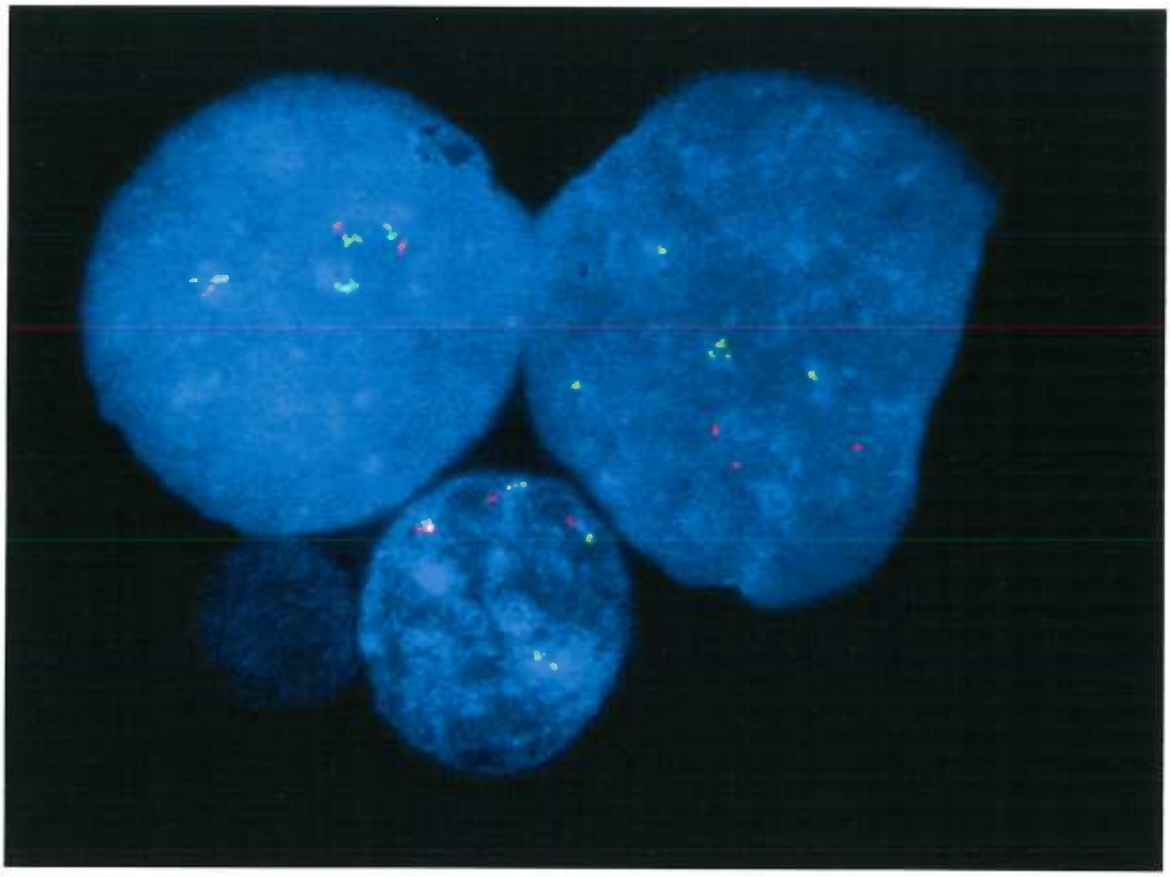

Figure 3.2. Loss of 1p demonstrated by interphase FISH. There are fewer signals for D1Z2 (red) compared to 1 alpha-satellite signals (green).

Establishing the timing of imprint switching in the germ line using *SNRPN* methylation patterns of pediatric germ cells tumors: implications for loss of imprinting in cancer

Kimberly J. Bussey[1,4], Helen J. Lawce[1], Eleanor Himoe[1,4], Xiao Ou Shu[2,4], Nyla A. Heerema[3,4], Susan B. Olson[1], and R. Ellen Magenis[1,4]

[1]Department of Molecular and Medical Genetics, Oregon Health Sciences University, Portland, Oregon

[2]Department of Pediatrics, University of South Carolina, Columbia, South Carolina

[3]Department of Genetics, Wayne Hughes Institute, Roseville, Minnesota

[4]Children's Cancer Group, Arcadia, California

Running Title: *SNRPN* Methylation in Pediatric GCTs

Corresponding Author:

R. Ellen Magenis, M.D.

Cytogenetic Research Laboratory

Oregon Health Sciences University

3181 SW Sam Jackson Park Rd., MP350

Portland, OR 97201

Tel: 503-494-2794

FAX: 503-494-6104

magenise@ohsu.edu

ABSTRACT

For imprinted genes, a mechanism must exist to switch the imprint from paternal to maternal or vice versa. The timing of imprint switching has been well established for one imprinted gene, *SNRPN*, in the mouse, however, it is not well characterized in the human. Germ cell tumors (GCTs) represent a way to begin to examine these events in the human germ line. An extrapolation from the mouse data can be made to predict the methylation pattern of *SNRPN* in GCTs based on tumor location in relationship to germ cell migration patterns. The resulting model predicts sacroccygeal tumors to have a methylated and unmethylated allele, ovarian tumors to have either unmethylated or methylated alleles, testicular tumors to have only unmethylated alleles, and anterior extragonadal tumors to have only unmethylated alleles. The methylation patterns of *SNRPN* in DNA from the cultured cells of 30 pediatric GCTs was examined and compared to the predicted methylation pattern for the hypothesized primordial germ cell (PGC) giving rise to the tumor. Eighteen tumors (6/7 sacroccocygeal, 9/11 ovarian, 1/3 testicular, 2/9 anterior extragonadal) had methylation patterns consistant with the model. The data demonstrate a similar pattern of demethylation and remethylation of *SNRPN* in the germ line between mice and humans. The data also support multiple etiologies for extragonadal GCTs in children and suggest a time frame for the initial events in GCT tumorigenesis.

INTRODUCTION

Genomic imprinting is the process of establishing a functional unequivalence between parental alleles, resulting in the complete or almost complete silencing of one allele. Imprinted genes have several characteristics in common, including clustering in specific regions throughout the genome which have sex-specific recombination frequencies (Pàldi et al. 1995), fewer exons compared to non-imprinted genes (Hurst and McVean 1996), the presence of short, direct repeats (Neumann et al. 1995), differences in the timing of allele replication (Izumikawa et al. 1991; Kitsberg et al. 1993; Gunaratne et al. 1995; Kawame et al.

1995; Lin et al. 1995), and parent-of-origin specific methylation patterns (Li et al. 1993). Imprinting was first experimentally suggested in a mammalian system by nuclear transfer experiments with male and female mouse pronuclei (Barton et al. 1984; McGrath and Solter 1984; Surani et al. 1984; Surani et al. 1986; Mann et al. 1995). Further evidence of imprinting resulted from analysis of parent-of-origin effects in the progeny of translocation carriers in the mouse (Cattanach and Kirk 1985). The study of the cytogenetically identical deletions in patients with the clinically different Prader-Willi and Angelman syndromes demonstrated a role for imprinting in human disease (Kaplan et al. 1987; Magenis et al. 1987; Donlon 1988; Knoll et al. 1989; Nicholls et al. 1989; Magenis et al. 1990).

Work with *H19* and *IGF2* in Wilms tumor has generated interest in the role of imprinted genes in carcinogenesis. The genes are located on the short arm of chromosome 11 at 11p15.5, a region shown to have loss of heterozygosity (LOH) in Wilms tumor. Paternal uniparental disomy of this region has been shown in the over-growth syndrome, Beckwith-Wiedemann, which includes an increased risk of developing tumors, including Wilms tumor. In most somatic tissues, *H19* is expressed solely from the maternal allele while *IGF2* is expressed from the paternal chromosome (Bartolomei et al. 1991; Rachmilewitz et al. 1992; Zhang and Tycko 1992; Giannoukakis et al. 1993; Ekström 1994). Biallelic expression of *IGF2* and loss of expression for *H19* have been reported in Wilms tumor (Rainier et al. 1993; Zhang et al. 1993; Moulton et al. 1994; Steenman et al. 1994; Taniguchi et al. 1995).

Perturbations of monoallelic expression of imprinted genes such as *H19* and *IGF2* have been described in a variety tumors, including adult testicular germ cell tumors (GCTs) (van Gurp et al. 1994; Mishina et al. 1996; Verkerk et al. 1997). However, unlike somatic cells, the primordial germ cells (PGCs) giving rise to GCTs may or may not have an imprint identical to the somatic imprint, with a corresponding change in gene expression, depending on their stage of development. This is because the imprint must be reset on one chromosome, paternal to maternal in female meiosis and maternal to paternal in male meiosis, such that the correct imprint is transmitted to offspring. These changes could cause confusion

when interpreting the results of imprinted gene analysis in GCTs. To determine whether there has been a change in imprinted gene expression in these tumors related to carcinogenesis, it is important to establish the "imprinting stage" of the PGC. Ideally, the gene(s) used for such a study would not be implicated in carcinogenesis, and the pattern of imprinting erasure and resetting of both the parental specific methylation patterns and gene expression would be established in the human germ line.

The gene *SNRPN* (small nuclear ribonucleoprotein associated polypeptide N) is a candidate for such a study. *SNRPN* encodes SmN, a protein involved in spliceosomes. The gene, located on chromosome 15q11-13 (chromosome 7 in the mouse), is imprinted and paternally expressed in somatic tissues (Leff et al. 1992; Reed and Leff 1994). The gene has 10 exons in humans and covers 25 kb of genomic DNA (Glenn et al. 1996). In addition, there are five untranslated exons 5' to exon 1 that are involved in transcripts found in brain, heart, testis, and ovary, and that are generated by alternative splicing with *SNRPN* 3' sequences. The resulting transcripts have only short reading frames and are unlikely to encode a protein (Dittrich et al. 1996). *SNRPN* has two regions of differential methylation in both humans and mice. The first, located in the 5' region of exon 1, is methylated on the maternal chromosome in all somatic tissues examined. The second region lies in intron 5 and is methylated on the paternal chromosome (Glenn et al. 1996; Shemer et al. 1997). An "imprinting center"(Buiting et al. 1995) lies in exon 1 of *SNRPN* (Sutcliffe et al. 1994; Buiting et al. 1995) and overlaps the area of differential methylation. It controls the imprinting of a 2Mb domain bordered by D15S63/PW71 and *SNRPN*.

In the mouse, it has been demonstrated that methylation in the 5' region of exon 1 is involved in regulation of expression (Szabo and Mann 1995a; Szabo and Mann 1995b; Shemer et al. 1997). This region is demethylated in primordial germ cells by the time these cells have migrated to the genital ridge at 12.5 days post-conception (dpc) (Shemer et al. 1997). Concurrent with the loss of methylation, expression is observed from both alleles (Szabo and Mann 1995b). Methylation is then reestablished in oocytes, while sperm remain

unmethylated (Shemer et al. 1997).

Although the pattern of methylation and demethylation of *SNRPN* exon 1 has not been established in the human germ line, extrapolation from the mouse data can be made to predict the methylation pattern of *SNRPN* in GCTs based on tumor location in relationship to germ cell migration patterns. Tumors located in the sacrum are postulated to have arisen from PGCs that had yet to reach the genital ridge and to erase their imprint. Therefore, these tumors will have a somatic methylation pattern in *SNRPN* exon 1 as seen on a Southern blot after digestion with a methylation-sensitive enzyme. GCTs in other extragonadal locations along the midline are speculated to have arisen from PGCs that erased the imprint on the maternal chromosome but failed to enter the genital ridge. The resulting tumors are expected to have only the unmethylated fragment present, with no evidence of a maternally derived methylated allele. GCTs in the gonads of males are predicted to have only the unmethylated fragment, regardless of the stage of PGC development since the male imprint in this region is to remain unmethylated. In the female germ line, the imprint is expected to be erased upon entrance into the genital ridge and reset to complete methylation of this region of *SNRPN* before the onset of meiosis. Therefore, tumors in female gonads are expected to have an unmethylated band if they arose from premeiotic germ cells and only a methylated band in GCTs from meiotic germ cells.

To test the hypothesis that *SNRPN* biology of the primordial germ cell is similar between the mouse and human, the methylation pattern of exon 1 of *SNRPN* was examined in pediatric GCTs. As a corollary to the hypothesis, the resulting data can be used to address the "imprinting stage" of GCTs in the pediatric population, based on tumor location, which may have an impact on study of the initiating events in tumorigenesis and the interpretation of studies of other imprinted genes.

METHODS

Sample Collection and Tissue Culture

Fresh tissue from pediatric germ cell tumors was collected as part of the collaborative Children's Cancer Group (CCG) protocols 8882, 8891, and E-22. Tumor tissue was mechanically and enzymatically dissociated and placed in short-term culture. Several sets of conditions were used for each tumor, sample size permitting. These included using three media: (1) Chang C (Irvine Scientific), (2) RPMI 1640 supplemented with 15% FBS, 2% L-glutamine, 1% insulin-transferrin-selenium, and 1% gentamicin, or (3) a 1:1 mixture of the two. In addition, cells were cultured on either plain or fibronectin-coated T-25 flasks. Six to 10 cultures were initiated from each sample. Samples were grown at 37°C in a 5% CO2 atmosphere. Cells from confluent flasks were collected for DNA extraction with phenol/ chloroform by standard methods.

Cytogenetic Analysis of Tumors

Harvests of tumor cells from the transport medium, using 3-hour Colcemid (50 μg/ ml) after overnight growth, methotrexate synchronization with 3-hour Colcemid exposure, or overnight Colcemid (30 μg/ ml) exposure, were done when possible. Cultures at 50% to 70% confluency, initiated as described above, were harvested at intervals up to 10 days to maximize the detection of abnormal cells while minimizing the percentage of normal fibroblasts. Metaphase cells were collected following either 3-hour Colcemid exposure or overnight Colcemid. Some cultures were synchronized with methotrexate prior to the 3-hour Colcemid exposure. After Colcemid, cells were trypsinized, collected, and incubated in 0.075 M KCl (pre-warmed at 37°C) for 10 to15 minutes. Cells were then fixed twice with 3:1 methanol:acetic acid and used to make slides immediately or stored at –20°C. Tumor cell suspensions were dropped on wet slides, air dried, and baked for 20 minutes at 90°C-95°C. Slides were GTW-banded. When possible, at least 20 cells were examined by microscope and photographed for each case. Heteromorphism analysis using quinacrine staining

to determine a possible meiotic origin was performed on teratomas from female patients (Overton et al. 1976; Olson et al. 1986). Ten to 20 cells were examined by microscope and serial photographic prints. In addition, G-band and/or Q-band heteromorphisms on chromosome 15 were examined, when possible, in other tumor types to confirm the biparental inheritance of the two homologs.

Methylation Assay

Two micrograms of genomic DNA from tumor cells in short-term culture was digested with *Xba*I and the methylation-sensitive enzyme, *Not*I, electophoresed through a 1.5% agarose gel and transferred to Biodyne B membranes. Additionally, DNA from peripheral leukocytes from a normal individual and from individuals with either Prader-Willi syndrome (PWS) or Angelman syndrome (AS) as a result of deletions of either the paternal (PWS) or maternal (AS) chromosome 15 was run on each gel as controls. Hybridizations were performed at 65°C overnight with a 600 bp *Not*I-*EcoR*I fragment (a gift from D. Driscoll) which included part of the 5'-untranslated region of *SNRPN* exon 1 (Glenn et al. 1996; Toth-Fejel et al. 1996). This probe detects a 4.3 kb band from the maternally derived methylated allele and a 0.9 kb band from the paternal unmethylated allele in normal somatic cells. Membranes were then visualized by autoradiography or on a phosphoimager.

RESULTS

To examine this model of imprint erasure and resetting at *SNRPN*, the methylation pattern of *SNRPN* exon 1 in short-term cell cultures of 30 GCTs from pediatric patients was determined. Eighteen of the 30 tumors had a methylation pattern consistent with the predictions based on tumor location. Six of seven tumors from the sacrococcygeal region had a methylation pattern similar to the somatic pattern, with both a 4.3 kb and a 0.9 kb fragment present (Figure 1 and Table 1). One sacrococcygeal tumor had only a 0.9 kb band present. Two of nine tumors from other extragonadal locations, one mediastinal tumor and one pineal

tumor, had only the 0.9 kb band present. The mediastinal tumor came from an individual with a constitutional chromosome abnormality (see Table 1). The remaining seven had a somatic methylation pattern.

Two of 11 ovarian tumors had only the 0.9 kb band present and six had only the 4.3kb band present. The other three had a somatic pattern. One of three testicular samples had only the 0.9 kb fragment present; the remaining two had a somatic pattern. One of these with a somatic pattern was demonstrated to have lost the previously observed chromosomally abnormal clone by G-band analysis (Bussey et al. 1999), and the result is probably indicative of the somatic methylation pattern expected from the outgrowth of supporting stroma. A dysgerminoma from the dysgenetic gonad of an XY female had only the 0.9 kb fragment.

The karyotypes of the GCTs were analyzed for clonal abnormalities by G-banding (see Table 1) (Bussey et al. 1999). In this analysis, it was determined that each tumor had at least two different homologs of chromosome 15 by examining G-band and/or Q-band heteromorphisms for chromosome 15, making the possibility unlikely that a nonsomatic methylation pattern at *SNRPN* was the result of a loss of one homolog.

DISCUSSION

The role of imprinted genes in cancer has not yet been clarified. Any study of alterations of imprinted gene expression in cancer needs to have knowledge of the expression and/or parent-of-origin specific methylation of the gene of interest in the tissue from which the tumor is derived. For most tumors, this involves comparing the tumor results to the known somatic pattern for the gene. However, GCTs represent a unique challenge for assessing changes in imprinted gene expression. This is because the cell giving rise to the tumor, the PGC, undergoes a developmentally regulated erasure and resetting of imprinted genes (Szabo and Mann 1995a; Szabo and Mann 1995b; Shemer et al. 1997; Huntriss et al. 1998). The expected pattern used for comparison in such a study of GCTs must take into

account the developmental stage of the PGC. This can be accomplished by examining an imprinted gene that is not involved in carcinogenesis and that has a well-characterized, stable methylation and/or expression pattern in the germ line.

SNRPN was used as such a gene to examine two questions: (1) Does the methylation of exon 1 follow a similar pattern of erasure and re-establishment in humans as it does with mice? and (2) Can the "imprinting stage" of the PGCs giving rise to GCTs in the pediatric population be established? These results support the hypothesis that the expected methylation patterns observed in GCTs can be based on the location of the tumor.

Based on the mouse model, tumors from the sacrococcygeal region would be expected to have arisen from a PGC that had not erased its imprint and would have a somatic methylation pattern. All but one of the sacrococcygeal tumors had results consistent with the predictions. The one sacrococcygeal tumor that had an unmethylated, 0.9 kb band present is of particular interest because it suggests that PGCs may erase the imprint at *SNRPN* before arriving at the genital ridge. Alternatively, this result may represent some other perturbation of PGC development such as mismigration out of the genital ridge after entry and imprint erasure.

Tumors from the ovary are predicted to be derived from germ cells which progressed to the genital ridge and should have erased the imprint of *SNRPN*, evidenced by a loss of methylation, and reset it to the maternal imprint by the time they have entered meiosis. GCTs in this location would be expected to have either an 0.9 kb or a 4.3 kb band present, but not both bands present. Three quarters of the ovarian tumors had results consistent with these predictions. Based on the model, tumors with only the 0.9 kb band are from premeiotic germ cells, while those with the 4.3 kb fragment alone are from germ cells that have entered meiosis. In support of this, three tumors determined to be a result of an error in meiosis II (cases 46, 39, and 29) (Bussey et al. 1999) have only the methylated, 4.3 kb band. Additional support for the model has been provided by Miura et al. (1999) who have shown increasing levels of *SNRPN* methylation with progression through meiosis in 25

benign ovarian teratomas.

Two of the three testicular tumors did not match the prediction that tumors from the testis would have only an unmethylated, 0.9 kb fragment. However, at least one of these tumors was known to have lost the original chromosomally abnormal clone in tissue culture. The result for this tumor, and possibly the other tumors that do not match the predictions, may represent the outgrowth of normal supporting stroma. The exact proportion of tumor to supporting stroma cultured for these studies is uncertain. Precise identification of tissue types is often difficult. Germ cell tumors often harbor foci of more than one histologic type, which may or may not be malignant but still part of the tumor. One possible way to reduce uncertainty would be to examine *SNRPN* methylation using frozen material that has been microdissected for the tumor alone.

The largest degree of discordance between the observed and the expected results occurred in the tumors from extragonadal locations other than sacrococcygeal. The model predicts that tumors from these other extragonadal locations would have only a 0.9 kb fragment in this assay because they arose from PGCs that had erased the imprint on the maternal chromosome but failed to enter the genital ridge. Only two of the nine tumors in this category had the expected methylation pattern based on the hypothesis; the other seven had a pattern consistent with somatic cells. While this could again represent outgrowth of normal supporting stroma or a disruption of developmental regulation of methylation at this locus, it may also be indicative of different etiologies for tumors from these areas. The question of whether GCTs from these locations arose from PGCs or totipotent embryonic stem cells remains unresolved (Castleberry et al. 1997). The results could suggest that GCTs arise from both types of cells, with those having only a 0.9 kb fragment coming from PGCs, while tumors with a somatic methylation pattern arose from an embryonic stem cell. What influence this might have on the tumor behavior remains to be seen.

The presence of constitutional chromosome abnormalities that lead to dysgenetic gonads also predisposes individuals to the development of GCTs, particularly in extragonadal

locations (Castleberry et al. 1997). Two of the three tumors from patients with constitutional chromosome abnormalities (cases 72, 88, and 92) had a methylation pattern consistent with the predictions of the model. The data suggest that while certain constitutional chromosome abnormalities disrupt the normal development of PGCs, possibly resulting in altered migration and/or tumor development, imprinting at *SNRPN* remains unperturbed.

In addition to supporting a similar pattern of imprint erasure and resetting between mice and humans, the data also shed light on the timing of events leading to GCTs in children. PGC migration begins at about four to six weeks of embryonic life. If environmental insults play a role in pediatric PGC development, the effects must be acting early in embryogenesis.

Previous work on imprinted genes in GCTs has focused on *H19* and *IGF2* because of their possible roles as tumor suppressor and oncogene, respectively. Three studies have examined *H19* expression in adult testicular germ cell tumors. All used RT-PCR to determine whether *H19* was expressed monoallelically or biallelically. In 10 seminomas and 10 nonseminomas, van Gurp et al. (1994) found that of 14 tumors informative for *H19*, 12 expressed both the maternal and paternal alleles. Similarly, 10 of 11 tumors informative for *IGF2* were biallelic. Seven tumors expressed both genes biallelically. In the tumors with monoallelic expression, LOH for the gene examined was observed. Mishina and colleagues (1996) observed similar results when investigating *H19* expression in 14 informative tumors; 11 had biallelic expression. Monoallelic expression was accompanied by LOH, which in one case was determined to be the maternal allele. In 1997, Verkerk et al. demonstrated biallelic expression of *H19* in 13 seminomas, seven of nine informative nonseminomas, and six carcinoma in situ.

Ross et al. (1999) examined the expression patterns of both *IGF2* and *H19* in a series of 61 pediatric GCTs from both gonadal and extragonadal sites. Eleven tumors were found to be informative for both genes. Of these, two of two testicular GCTs, three of five ovarian GCTs , and one of four extragonadal GCTs expressed both genes biallelically. The remaining tumors had a variety of expression patterns with either *H19* or *IGF2* showing biallelic

expression; the only exception was one ovarian tumor that retained monoallelic expression for both genes.

All of these studies have shown that the expression patterns of *IGF2* and *H19* are not what is expected for somatic cells. The question remains, however, whether the biallelic expression of one or both genes is a function of the carcinogenic process, or whether the biallelic expression is instead a reflection of the PGC giving rise to the tumor. This does not preclude an involvement of biallelic expression of *IGF2* or *H19* in the etiology of GCTs. However, the biallelic expression may be a normal part of PGC development. Beginning to answer this question would require two steps: a good analysis of gene expression in the primordial germ line of the human for both *H19* and *IGF2* and a knowledge of the "imprinting stage" of the PGC precursor of the tumor.

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Table 4.1: *SNRPN* Methylation Results and Cytogenetic Analysis of 30 Pediatric GCTs

Case	Age[b]	Sex[c]	Tumor Location	Tumor Histology[d] And Clinical Stage	Predicted SNRPN Result	Observed SNRPN Result	Cytogenetic Results[e]
23[a]	1.4 y	F	Sacrococcygeal	EST	0.9 kb and 4.3 kb bands	0.9 kb and 4.3 kb bands	90<4n>,XX, del(X)(p22)x2,-4,-5,-9, -17,-17,-19,-20, +der(?)t(?;1)?;q12)x2,+ mar1,+mar2,+mar3[15] /46,XX[5]
8[a]	5 d	F	Sacrococcygeal	IT, grade 3/ EST Stage I	0.9 kb and 4.3 kb bands	0.9 kb and 4.3 kb bands	46,XX[24]
19[a]	7 m	F	Sacrococcygeal	EST Stage IV	0.9 kb and 4.3 kb bands	0.9 kb and 4.3 kb bands	46,XX[20]
92	4 d	F	Sacrococcygeal	MT/IT, grade 1 Stage I	0.9 kb and 4.3 kb bands	0.9 kb and 4.3 kb bands	46,XX[22]
24[a]	1.4 y	F	Sacrococcygeal	MT/EST	0.9 kb and 4.3 kb bands	0.9 kb and 4.3 kb bands	46,XX[20]
97	11 d	F	Sacrococcygeal	MT/EST	0.9 kb and 4.3 kb bands	0.9 kb and 4.3 kb bands	46,XX[19]
10[a]	7 d	F	Sacrococcygeal	MT	0.9 kb and 4.3 kb bands	0.9 kb band	46,XX[21]
28[a]	2 y	F	Neck	MT	0.9 kb band	0.9 kb and 4.3kb bands	46,XX[20]
72[a]	10 y	M	Mediastinal	MT/EST/EC/ CC Stage II	0.9 kb band	0.9 kb band	48,XXYc,+21[9]/ 49,XXYc,+17,+21[9]
70[a]	9 y	M	Pineal	U	0.9 kb band	0.9 kb band	49,XY,+X,+i(12)(p10), +21[19]
84	11 y	M	Pineal	GE	0.9 kb band	0.9 kb and 4.3 kb bands	46,XY[15]
15[a]	2 m	F	Midline CNS	MT	0.9 kb band	0.9 kb and 4.3 kb bands	46,XX[22]
83	14 y	M	Thalamus	GE	0.9 kb band	0.9 kb and 4.3 kb bands	46,XY[13]/ 92,XXYY[6]
98	1.17 y	F	Retroperitoneal	MT	0.9 kb band	0.9 kb and 4.3 kb bands	46,XX[14]

99	13 y	M	Brain	U	0.9 kb band	0.9 kb and 4.3 kb bands	46,XY[18]
5[a]	3 d	F	Pelvis	MT	0.9 kb band	0.9 kb and 4.3 kb bands	46,XX[22]
31[a]	7 y	F	Ovary	IT/EST Stage I	Premeiotic: 0.9 kb band Meiotic: 4.3 kb band	0.9 kb and 4.3 kb bands	47,XX,+2[5]/ 47,XX,+3[6]/ 48,XX,+2,+3[5]/ 49,XX,+2,+3,+14[4]
46[a]	14 y	F	Ovary	MT	Premeiotic: 0.9 kb band Meiotic: 4.3 kb band	4.3 kb band	(23,X)x2,+8[15]
88	13 y	F	Ovary	GE	Premeiotic: 0.9 kb band Meiotic: 4.3 kb band	0.9 kb band	46,XX[36]
87	2 y	F	Ovary	IT, grade 2/3	Premeiotic: 0.9 kb band Meiotic: 4.3 kb band	4.3 kb band	47,XX,+mar[2]/ 46,XX[15]
91	16 y	F	Ovary	MT	Premeiotic: 0.9 kb band Meiotic: 4.3 kb band	0.9 kb and 4.3 kb bands	46,XX[17]
39[a]	10y	F	Ovary	IT	Premeiotic: 0.9 kb band Meiotic: 4.3 kb band	4.3 kb band	(23,X)x2[31]
36[a]	10y	F	Ovary	MT	Premeiotic: 0.9 kb band Meiotic: 4.3 kb band	4.3 kb band	47,XX,+7[20]
82	6 y	F	Ovary	IT, grade 1	Premeiotic: 0.9 kb band Meiotic: 4.3 kb band	4.3 kb band	45,XX,-16[5]/ 46,XX[15]
29[a]	6y	F	Ovary	MT	Premeiotic: 0.9 kb band Meiotic: 4.3 kb band	4.3 kb band	(23,X)x2[20]
86	12 y	F	Ovary	U	Premeiotic: 0.9 kb band Meiotic: 4.3 kb band	0.9 kb and 4.3 kb bands	90<4N>,XXX,-X, i(1)(q10),-3,-4, i(4)(q10),-5,+8,+8, der(9)inv(9)(p11q12) del(9)(q22),-13,-16,-18, +20,+21,+21[4]/ 92,XXXX[5]/ 46,XX[11]

79[a]	17y	M	Testis	EC/SE/TM	Only 0.9 kb band	0.9 kb and 4.3 kb bands (clone lost in culture)	60-68<3n>,XXY,-4,-9, -10,-11,+der(12) ?t(12;12)(pter-q13:p11.2-pter)x2,-13, -16,-18,-22 [cp7]/60-68, idem,del(6)(q?13q31) [cp8]/46,XY[4]
81[a]	18y	M	Testis	MT/EC/CC	Only 0.9 kb band	0.9 kb band present	66-70<3n>,XXY, der(1)t(1;5)(p11;p11), +der(1)t(1;11) (p?36;q11),-4,-6,+7,+7, dic(9;11)(p?22;q?23), +dic(9;11) (p?22;q?23), -10,-11,-11, add(12)(q?13), dic(12;16)(p11;q?24), +i(12)(p10),+14,-16, -17,-18 der(19)t(?17;19) (q11;q?13.1),+21,inc [cp20]
95	16 y	M	Testis	U	Only 0.9 kb band	Both bands present	48,XXYc,+21[1]/ 48,XXYc,+X[1]/ 47,XXYc[17]
89	17 y	F	Gonad	GE	Only 0.9 kb band	0.9 kb band present	46,XY[17]

[a]Cytogenetic results published previously (Bussey et al., 1999).
[b]d, day; w, week; y, year.
[c]Phenotypic Sex.
[d]MT=mature teratoma; IT=immature teratoma; TM= malignant teratoma; EST=endodermal sinus tumor; EC=embryonal carcinoma; GE=germinoma; CC=choriocarcinoma; MXD=mixed malignant germ cell tumor (tumor has two or more histologies indicative of malignancy); U=Unknown.
[e]Cytogenetic analysis done by Helen Lawce for cases 23, 8, 19, 24, 10, 28, 72, 70, 15, 5, 31, 46 (KB did Q-band analysis), 39, 36, 29 (KB did Q-band analysis), 79, and 81. Eleanor Himoe did the analysis of cases of 91 and 92.

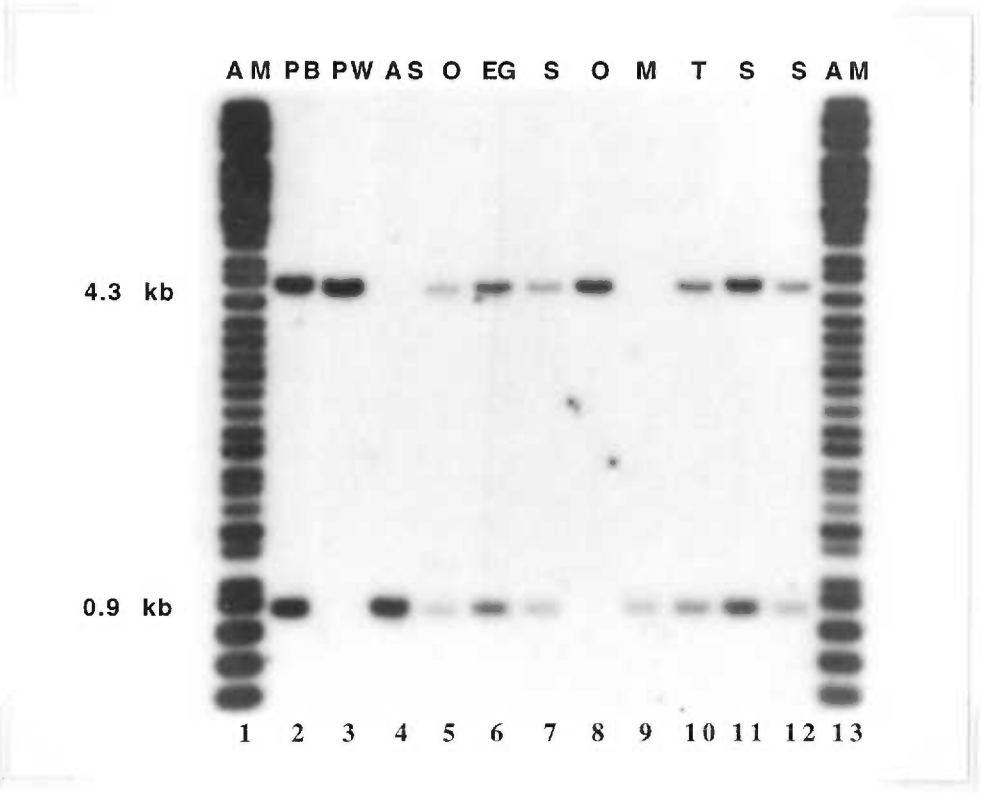

Figure 4.1: Methylation Patterns of Exon 1 in *SNRPN* in GCTs
Southern blot of DNA from GCTs and controls after *XbaI*/*Not*I dual digest. In somatic cells, the digest yields a maternally derived, 4.3 kb methylated fragment and a paternally derived 0.9 kb unmethylated fragment (lane 2). **AM**, DNA Analysis Marker (Gibco BRL); **PB**, normal peripheral blood; **PW**, peripheral blood of a Prader-Willi syndrome patient; **AS**, peripheral blood of an Angelman syndrome patient; **O**, ovary; **EG**, extragonadal (neck); **S**, sacrococcygeal; **M**, mediastinal; **T**, testis

SNRPN methylation patterns in frozen adult and pediatric germ cell tumors: determining the timing of the initiation of tumor formation

Kimberly J. Bussey[1,4], Eleanor Himoe[1,4], Xiao Ou Shu[2,4], Elizabeth Perlman[3,4], Susan B. Olson[1], and R. Ellen Magenis[1,4]

[1]Department of Molecular and Medical Genetics, Oregon Health Sciences University, Portland, Oregon

[2]Department of Pediatrics, University of South Carolina, Columbia, South Carolina

[3]Department of Pathology, Johns Hopkins University, Baltimore, Maryland

[4]Children's Oncology Group, Arcadia, California

Corresponding Author:

R. Ellen Magenis, M.D.

Cytogenetics Research Laboratory

Oregon Health Sciences University

3181 SW Sam Jackson Park Rd., MP 350

Portland, OR 97201

Tel: 503-494-2794

FAX: 503-494-6104

magenise@ohsu.edu

ABSTRACT

The etiology of human germ cell tumors (GCTs) remains unknown. Several lines of evidence suggest that GCTs can be grouped by patient sex, age, and tumor location. These groupings may reflect important differences in etiology that might influence clinical behavior. Previous work, using tissue cultures from pediatric GCTs, suggests that the methylation pattern of the imprinted *SNRPN* gene is indicative of the "imprinting stage" of the primordial germ cell (PGC) giving rise to the tumor. Because of questions regard the use of cultured material, this study examined the methylation pattern of *SNRPN* in frozen tissue samples of 33 adult testicular GCTs, 2 adult mediastinal GCTs, and 20 pediatric GCTs from various locations. Five of 33 adult testicular GCTs and the 2 adult mediastinal GCTs had only the unmethylated 0.9 kb band on Southern blot after a dual digest with *Xba*I and *Not*I. In an additional 16 tumors both the methylated, 4.3 kb and the 0.9 kb band were present, with the 0.9 kb band significantly more intense. Among the pediatric GCTs, four of five of tumors from sacral/retroperitoneal locations had both bands present. Nine of 16 ovarian GCTs had either the 0.9 kb band or the 4.3 kb band alone; an additional five tumors had both bands, but the 0.9 kb band was more intense. Of the two GCTs from extragonadal locations, one had both bands, with the 0.9 kb band more intense. The data add further evidence to support the model of imprint erasure and resetting established in the mouse, and suggest the events leading to GCT formation act on PGCs as early in embryonic development as 24 days post conception.

INTRODUCTION

Germ cell tumors (GCTs) present in humans in both gonadal and extragonadal locations, usually along the midline. GCTs can present in children and adults, with almost half to 2/3 of GCTs in children being extragonadal (Harms and Janig 1986), while the overwhelming majority of adult GCTs are located in the gonads. The presentation of GCTs in both chil-

dren and adults can range from benign to malignant, and resemble germ cells (seminomas, dysgerminomas, and germinomas), or somatic cells with differentiation through either embryonic (mature or immature teratomas) or extraembryonic (embryonal carcinoma, endodermal sinus tumors/ yolk sac tumors, or choriocarcinoma) pathways (Castleberry et al. 1997). However, the frequency of specific histologic types, anatomical location, clinical behavior, and natural history of GCTs differ between children and adults (Harms and Janig 1986).

Several possible risk factors have been explored for ties to GCT formation, primarily in relationship to the presentation of adult testicular GCTs. Those associated with adult testicular GCTs include gonadal dysgenesis, particularly related to constitutional chromosome abnormalities (Scully 1970; Schellhas 1974; Sogge et al. 1979; Curry et al. 1981; Turner et al. 1981; Mann et al. 1983; Muller et al. 1985; Nichols et al. 1987; Hasle et al. 1992; Derenoncourt et al. 1995; Dieckmann et al. 1997; Satge et al. 1998); cryptorchidism; maternal exogenous hormonal use during pregnancy; radiation exposure; and childhood viral infections (e.g. mumps) (Shu et al. 1993). A preliminary study of risk factors for pediatric GCTs suggests parental exposure to chemical solvents, plastic or resin fumes, increased birthweight for gestational age, prolonged breastfeeding, and maternal urinary tract infections may increase the risk of developing GCTs (Shu et al. 1993).

Currently, studies are underway through the Children's Oncology Group to follow up on the preliminary work of Shu et al. (1993). As part of those studies, the pattern of methylation of the 5'-untranslated region of the imprinted gene, *SNRPN*, was examined in the short-term tissue cultures of pediatric tumors received for cytogenetic analysis. The data collected supported a shared model of imprint erasure and re-establishment, as evidenced by methylation patterns, between mice and humans (Bussey et al, unpublished data).

In this model, GCTs from the sacrococcygeal region are suspected to have arisen from PGCs that had yet to erase their imprint. Therefore, these tumors will have a somatic methylation pattern in *SNRPN* exon 1 as seen on a Southern blot after digestion with a

methylation-sensitive enzyme. GCTs in other extragonadal locations along the midline are speculated to have arisen from PGCs that erased the imprint on the maternal chromosome but failed to enter the genital ridge. The resulting tumors are predicted to have only the unmethylated fragment present, with no evidence of a maternally derived methylated allele. Since the male imprint in this region is to remain unmethylated, GCTs in the gonads of males are expected to have only the unmethylated fragment, regardless of the stage of PGC development. In the female germ line, the imprint is expected to be erased upon entrance into the genital ridge and reset to complete methylation of this region of *SNRPN* before the onset of meiosis. Therefore, tumors in female gonads are expected to have an unmethylated band if they arose from premeiotic germ cells and only a methylated band in GCTs from meiotic germ cells.

By establishing the "imprinting stage" of the PGC giving rise to the tumor, the timing of events leading to tumor formation can be refined. To this end, the pattern of *SNRPN* methylation was determined in frozen tissue samples of 33 adult testicular GCTs, both primary and metastases, 2 adult mediastianl GCTs, and 20 pediatric GCTs. The results suggest that events early in embryonic life may play a role in the genesis of GCTs.

METHODS

Sample procurement

Approximately 0.5 cm^3 piece of snap frozen tissue from 33 adult testicular and 2 adult mediastinal GCTs was obtained from the Indiana University Tissue Procurement lab and stored at -70°C. Nine samples of pediatric GCTs were set aside from material received for G-band analysis, frozen in liquid N_2, and stored at –70°C. The tissue was mechanically minced, placed in RNase digestion buffer (10 mM Tris, pH 8.0; 0.1 M EDTA, pH 8.0; 0.5% SDS) with proteinase K, and incubated overnight at 37°C. The DNA was extracted with an equal volume of phenol, followed by extraction with a 24:1 chloroform:isoamyl alcohol.

The DNA was ethanol precipitated using 3 M sodium acetate, dried, and resuspended in TE, pH 8.0. The DNA from an additional 11 pediatric GCTs was obtained from frozen tumor specimens collected in the laboratory of E. Perlman at Johns Hopkins University.

Assaying *SNRPN* Methylation Patterns

Two micrograms of genomic DNA from frozen tumor tissue was digested with *Xba*I and the methylation-sensitive enzyme, *Not*I, electophoresed through a 1.5% agarose gel and transferred to Biodyne B membranes. Additionally, DNA from peripheral leukocytes from a normal individual was run on each gel as a control. Hybridizations were performed at 65°C overnight with a 600 bp *Not*I-*Eco*RI (a gift from D. Driscoll) fragment that included part of the 5'-untranslated region of *SNRPN* exon 1 (Glenn et al. 1996; Toth-Fejel et al. 1996). This probe detects a 4.3 kb band from the maternally derived methylated allele and a 0.9 kb band from the paternal unmethylated allele in normal somatic cells. A probe for *FES* (15q26) was also hybridized as a control for DNA loading between lanes. Membranes were then visualized on a phosphoimager.

The results were quantified using ImageQuant, v1.0 (Molecular Dynamics). The quantified results were then used to calculate a ratio of the intensity of 4.3 kb band to the sum of the intensities of both the 4.3 kb and 0.9 kb bands of *SNRPN*. A tolerance interval for a 95% confidence of 95% of the data being contained within the bounds was constructed based on the data from the normal controls. Only those tumors with ratios outside of these limits were determined to have unequal intensities between the 0.9 kb and 4.3 kb bands.

Microsatellite Determination

DNA from tumors that had only one band Southern analysis of *SNRPN* methylation was amplified for D15S165 and D15S822 (primers kindly provided by M. Litt, available from Research Genetics,). 50 ng of DNA was amplified by PCR (Tris, pH , 1.5mM MgCl2, 200 μM dNTPs, 0.5 μM forward and reverse primers, and 0.625 U Amplitaq (PE Biosystems)). Reactions were run as follows: one cycle of 94°C for 5 minutes, 15 cycles of 94°C for 15

seconds, touch down 30 seconds 65°C to 50°C with –1°C per cycle, 72°C for 30 seconds, 25 cycles of 94°C for 15 sec., 50°C for 30 sec., and 72°C for 30 sec., followed by a final 5 minute extension at 72°C. Products were checked on an agarose gel and then run out on a 6% denaturing polyacrylamide gel and transferred to Biodyne B membranes. Products were then visualized by hybridization with a digoxigenin-labeled oligonucleotide followed by colorimetric detection using anti-digoxigenin antibody conjugated to alkaline phosphatase to catalyze the reaction between NBT and BCIP.

RESULTS

To investigate whether the cellular origin of testicular GCTs differed between children and adults, 33 samples of adult testicular GCTs and 2 mediastinal GCTs were obtained from the tissue bank of the Indiana University Tissue Procurement Laboratory. All samples had been snap frozen and dissected for tumor tissue alone. Twelve samples were primary tumors from testis; the remaining samples were from various metastatic sites (see Table 1). The *SNRPN* methylation pattern was analyzed and the results quantified on a phosophoimager. Seven tumors (5 testicular and 2 mediastinal) showed complete loss of the 4.3 kb methylated fragment, in agreement with the model (figure 5.1). An additional 16 tumors had both bands present, but the unmethylated, 0.9 kb band was significantly more intense. The remaining 12 tumors had both the 4.3 kb and 0.9 kb bands of equal intensity. There was a correlation of the methylation pattern and whether the site of the tumor was primary or metastatic, such that 9 of the 12 tumors with both bands present at equal intensities were from metastatic sites (8 from lymph nodes and 1 from the lung, Table 5.1). The remaining three tumors from this group were testicular, two seminomas and one of mixed histologic types.

To rule out the possibility that tumors with only the 0.9 kb fragment present were a result of the loss of one homolog of chromosome 15, two polymorphic markers near the centromere of chromosome 15, D15S165 and D15S822, were amplified by PCR and

analyzed on a 6% denaturing polyacrylamide gel. All seven tumors demonstrated at least one marker with two alleles, confirming the presence of two different homologs of chromosome 15.

In addition to the adult samples, pediatric GCTs were also examined (figure 5.2), with special focus on malignant ovarian tumors (see Table 5.2 for details). Four of five tumors from sacrococcygeal or retroperitoneal locations had the 4.3 kb and 0.9 kb bands. One tumor had only the 4.3 kb band. Of 16 GCTs from the ovary, 6 had the 0.9 kb band only and 3 had the 4.3 kb band only. Five of the 16 had both bands present, but the unmethylated, 0.9 kb band was more intense. Two tumors had both bands present and equally intense. Two tumors from extragonadal locations, predicted to have the 0.9 kb band alone, had both bands present. In one of these, the 0.9 kb band was more intense. As with the adult tumors, all tumors displaying only one band were examined by PCR for the presence of two homologs. All tumors examined had evidence for two homologs of chromosome 15.

DISCUSSION

The timing of the initiating events leading to the development of GCTs is important in the search for risk factors and preventive strategies. The current feeling in the literature is that the initial events leading to tumor development occur early, sometime during fetal life (Castleberry et al. 1997). This study examined the methylation pattern of the 5' untranslated region the imprinted gene SNRPN to establish the "imprinting stage" of the PGC precursor of the GCT. Because the expected methylation pattern can be predicted at this locus for a tumor in a given location, and the relationship between this pattern and the timing of the events during development is known, a time reference for the initiating events leading to tumor formation can be established.

Frozen tissue from adult testicular tumors was examined to determine if PGCs at similar

stages of development are involved in the testicular tumors from children and adults. The tumors are expected to have only an unmethylated band, regardless of whether the PGC has entered meiosis or not. This limits the determination of the precursor identity to a PGC that had yet to demethylate the maternal allele or a somatic cell and a precursor which had erased the imprint and demethylated the maternal allele. Twenty-three of 35 tumors had methylation patterns indicative of a precursor that had erased the imprint at *SNRPN* and demethylated the maternal allele. Seven of 35 adult male tumors examined had only an unmethylated fragment present. Sixteen tumors had evidence of a more intense 0.9 kb band as compared to the 4.3 kb band, suggesting a population of cells with the 0.9 kb band alone in a background of normal somatic cells. It is unlikely that this result represents simply an overrepresentation of the paternal homolog of chromosome 15 because the tolerance interval constructed to determine whether the imbalance in intensities is significant is too broad to permit detection of an imbalance of two paternal homologs to one maternal homolog. Most adult testicular GCTs are near-triploid, but gain of 15 has not been reported as a common finding by cytogenetics or comparative genomic hybridization (de Jong et al. 1990; Suijkerbuijk et al. 1994; Mostert et al. 1996; Sandberg et al. 1996; de Jong et al. 1997; Summersgill et al. 1998). Even though the tumors were dissected for tumor material, this was done on a gross basis, not on a cell by cell basis. Normal somatic cell contamination could come from infiltrating lymphocytes, recruited vascular elements, or microscopically small areas of normal tissue; GCTs are often a mix of benign and malignant components that can be difficult to discriminate from normal tissue.

However, twelve tumors had both the 4.3 kb and 0.9 kb band in equal intensity, at variance with the expected pattern of methylation. While this may be indicative of a non-germ cell origin for these tumors, it is likely this represents normal cell contamination. Nine of these 12 tumors were from metastatic locations. Such tumors might be expected to have more normal cell contamination.

Miura and colleagues (1999) observed unequal intensities between the methylated and

unmethylated bands in their study of benign ovarian teratomas. In two tumors where they analyzed both frozen material and tissue culture material, the frozen material demonstrated a more intense methylated band, while the tissue cultured cells had only the methylated band. In the one tumor in this study for which there is also tissue culture data, the findings are reversed. The tissue culture data showed two bands of equal intensity, while the frozen tissue data results were of a single unmethylated band. This supports the presence of two cell populations in the tumors that are most likely explained as tumor and normal somatic cells.

Based on the mouse model, tumors from the sacrococcygeal region would be expected to have arisen from a PGC that had not erased its imprint and would have a somatic methylation pattern. All but one of the sacrococcygeal tumors had results consistent with the predictions. One sacrococcygeal tumor had an methylated, 4.3 kb band present. This result may represent mismigration out of the developing ovary after entry to the genital ridge, imprint erasure and imprint reestablishment. The tumor may also be a metastasis of a tumor from a primary site in the ovary.

Tumors from the ovary are predicted to be derived from germ cells which progressed to the genital ridge and should have erased the imprint of *SNRPN*, evidenced by a loss of methylation, and reset it to the maternal imprint prior to entering meiosis. GCTs in this location would be expected to have either an 0.9 kb or a 4.3 kb band present, but not both bands present. 86% of the ovarian tumors had results consistent with these predictions. Based on the model, tumors with only the 0.9 kb band are from premeiotic germ cells, while those with the 4.3 kb fragment alone are from germ cells that have entered meiosis.

Finally, the model predicts that tumors from these other extragonadal locations would have only a 0.9 kb fragment in this assay because they arose from PGCs that had erased the imprint on the maternal chromosome but failed to enter the genital ridge. Both of the two tumors from extragonadal locations had the 4.3 kb and 0.9 kb bands present, but in one the 0.9 kb band was more intense. This provides further support for multiple different etiologies

for tumors from these areas. In previous work with cultured cells, only 2 of 9 tumors had the expected results (Bussey et al., unpublished data). The question of whether GCTs from these locations arose from PGCs or totipotent embryonic stem cells remains unresolved (Castleberry et al. 1997). The results could suggest that GCTs arise from both types of cells, with those having only a 0.9 kb fragment coming from PGCs, while tumors with a somatic methylation pattern arose from an embryonic stem cell. What influence this might have on the tumor behavior remains to be seen.

The data suggest that the events leading to tumorigenesis are acting upon PGCs as early as 24 days of fetal life (the time at which PGCs start migrating to the genital ridge). In this context, events leading to the presentation of congenital sacral GCTs are possibly directly oncogenic. However, tumors arising later in childhood could possibly originate from a PGC that sustains a predisposing mutation in the early time frame. The initial event might alter PGC development that then predisposes the germ cell to tumor development. The presence of constitutional chromosome abnormalities that lead to dysgenetic gonads may be this predisposing hit. Tumors presenting in the adult testis are likely to be derived from PGCs that sustain an initiating event at some point after germ cell migration into the genital ridge, suggesting a time frame for initiation anywhere from the sixth or seventh week of embryonic life to adolescence or later.

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Table 5.1: *SNRPN* Methylation Patterns in 35 Adult Male GCTs

Case	Location	Histology	Predicted Result	Observed Result
A1	Para-nephric soft tissue	EST	0.9 kb band only	Both bands present, 0.9 kb band more intense
A2	Lymph node	SE	0.9 kb band only	Both bands present
A3	Lymph node	MXD	0.9 kb band only	Both bands present, 0.9 kb band more intense
A4	Mediastinum	MT/EC	0.9 kb band only	0.9 kb band only
A5	Lymph node	EC	0.9 kb band only	Both bands present
A6	Lymph node	EC	0.9 kb band only	Both bands present, 0.9 kb band more intense
A7	Testis	SE	0.9 kb band only	Both bands present
A8	Lymph node	EC	0.9 kb band only	Both bands present, 0.9 kb band more intense
A9	Lymph node	MXD	0.9 kb band only	Both bands present
A10	Pelvic soft tissue	MXD	0.9 kb band only	0.9 kb band only
A11	Testis	SE	0.9 kb band only	Both bands present
A12	Lymph node	EC	0.9 kb band only	Both bands present, 0.9 kb band more intense
A13	Lymph node	EC	0.9 kb band only	Both bands present
A14	Lymph node	MXD	0.9 kb band only	Both bands present
A15	Lymph node	EC	0.9 kb band only	Both bands present
A16	Lymph node	EC	0.9 kb band only	0.9 kb band only
A17	Testis	EC	0.9 kb band only	Both bands present, 0.9 kb band more intense
A18	Lymph node	EC	0.9 kb band only	0.9 kb band only
A19	Testis	MXD	0.9 kb band only	0.9 kb band only
A20	Testis	MT/EC	0.9 kb band only	Both bands present, 0.9 kb band more intense
A21	Testis	SE	0.9 kb band only	Both bands present, 0.9 kb band more intense
A22	Testis	SE	0.9 kb band only	Both bands present, 0.9 kb band more intense
A23	Mediastinum	EST	0.9 kb band only	0.9 kb band only
A24	Lymph node	EC	0.9 kb band only	Both bands only
A25	Liver	EST	0.9 kb band only	Both bands present, 0.9 kb band more intense
A26	Lymph node	EC	0.9 kb band only	Both bands present, 0.9 kb band more intense
A27	Testis	MT/EC	0.9 kb band only	Both bands present, 0.9 kb band more intense
A28	Testis	MXD	0.9 kb band only	Both bands present

A29	Testis	SE	0.9 kb band only	Both bands present, 0.9 kb band more intense
A30	Lung	CC	0.9 kb band only	Both bands present
A31	Lymph node	MXD	0.9 kb band only	Both bands present, 0.9 kb band more intense
A32	Testis	MXD	0.9 kb band only	Both bands present, 0.9 kb band more intense
A33	Lymph node	EC	0.9 kb band only	Both bands present
A34	Lymph node	EST	0.9 kb band only	0.9 kb band only
A35	Testis	SE	0.9 kb band only	Both bands present, 0.9 kb band more intense

MT, mature teratoma; SE, seminoma; EST, endodermal sinus tumor; EC, embryonal carcinoma; CC, choriocarinoma; MXD, mixed germ cell tumor

Table 5.2: *SNRPN* Methylation Patterns in 20 Frozen Pediatric GCTs

Case[a]	Location	Histology	Predicted Result	Observed Result
103	Retroperitoneal	MT	Both 4.3 kb and 0.9 kb bands present	Both bands present
97	Sacrum	U	Both 4.3 kb and 0.9 kb bands present	Both bands present
98	Retroperitoneal	MT	Both 4.3 kb and 0.9 kb bands present	Both bands present
105	Sacrum	EST	Both 4.3 kb and 0.9 kb bands present	4.3 kb band only
93	Pelvis	MT	0.9 kb band only	Both bands present, 0.9 kb band more intense
99	Brain	U	0.9 kb band only	Both bands present
85	Ovary	DYS	Premeiotic: 0.9 kb band only Meiotic: 4.3 kb band only	0.9 kb band only
86	Ovary	DYS	Premeiotic: 0.9 kb band only Meiotic: 4.3 kb band only	0.9 kb band only
87	Ovary	IT	Premeiotic: 0.9 kb band only Meiotic: 4.3 kb band only	0.9 kb band only
90	Ovary	TM	Premeiotic: 0.9 kb band only Meiotic: 4.3 kb band only	Both bands present, 0.9 kb band more intense
109	Ovary	DYS	Premeiotic: 0.9 kb band only Meiotic: 4.3 kb band only	Both bands present, 0.9 kb band more intense
110	Ovary	DYS	Premeiotic: 0.9 kb band only Meiotic: 4.3 kb band only	0.9 kb band only
112	Ovary	DYS	Premeiotic: 0.9 kb band only Meiotic: 4.3 kb band only	Both bands present
113	Ovary	DYS	Premeiotic: 0.9 kb band only Meiotic: 4.3 kb band only	Both bands present
114	Ovary	DYS	Premeiotic: 0.9 kb band only Meiotic: 4.3 kb band only	0.9 kb band only
115	Ovary	DYS	Premeiotic: 0.9 kb band only Meiotic: 4.3 kb band only	0.9 kb band only
116	Ovary	EST	Premeiotic: 0.9 kb band only Meiotic: 4.3 kb band only	Both bands present, 0.9 kb band more intense
118	Ovary	EST	Premeiotic: 0.9 kb band only Meiotic: 4.3 kb band only	4.3 kb band only
119	Ovary	O	Premeiotic: 0.9 kb band only Meiotic: 4.3 kb band only	Both bands present, 0.9 kb band more intense
120	Ovary	O	Premeiotic: 0.9 kb band only Meiotic: 4.3 kb band only	0.9 kb band only

[a]Cases 109-113 done by Eleanor Himoe

[b]MT, mature teratoma; IT, immature teratoma; EST, endodermal sinus tumor; DYS, dysgerminoma; TM, malignant teratoma; O, other malignant GCT (not EST or DYS); U, unknown

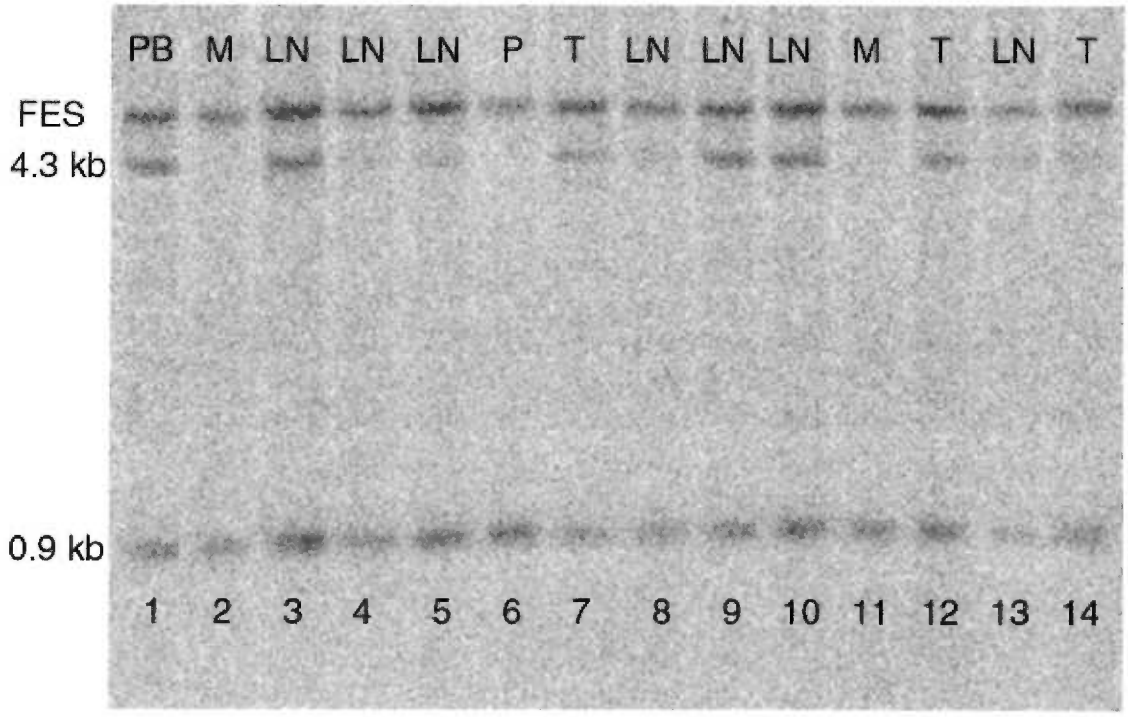

Figure 5.1: *SNRPN* Methylation Patterns in Frozen Adult Male Tumor Samples
DNA was extracted from flash frozen tumor samples, digested with *Xba*I and *Not*I, and probed to detect the methylation pattern at the 5'UTR of *SNRPN*. Normal somatic cells yield a maternally derived, methylated 4.3 kb band and a paternally derived, unmethylated 0.9 kb band. An additional probe for *FES* (15q26) was included as a control for gel loading. All but one sample in this figure are adult testicular GCTs. **PB**, normal peripheral blood; **M**, mediastinal GCT; **LN**, lymph node metastasis; **P**, pelvic metastasis; **T**, testis

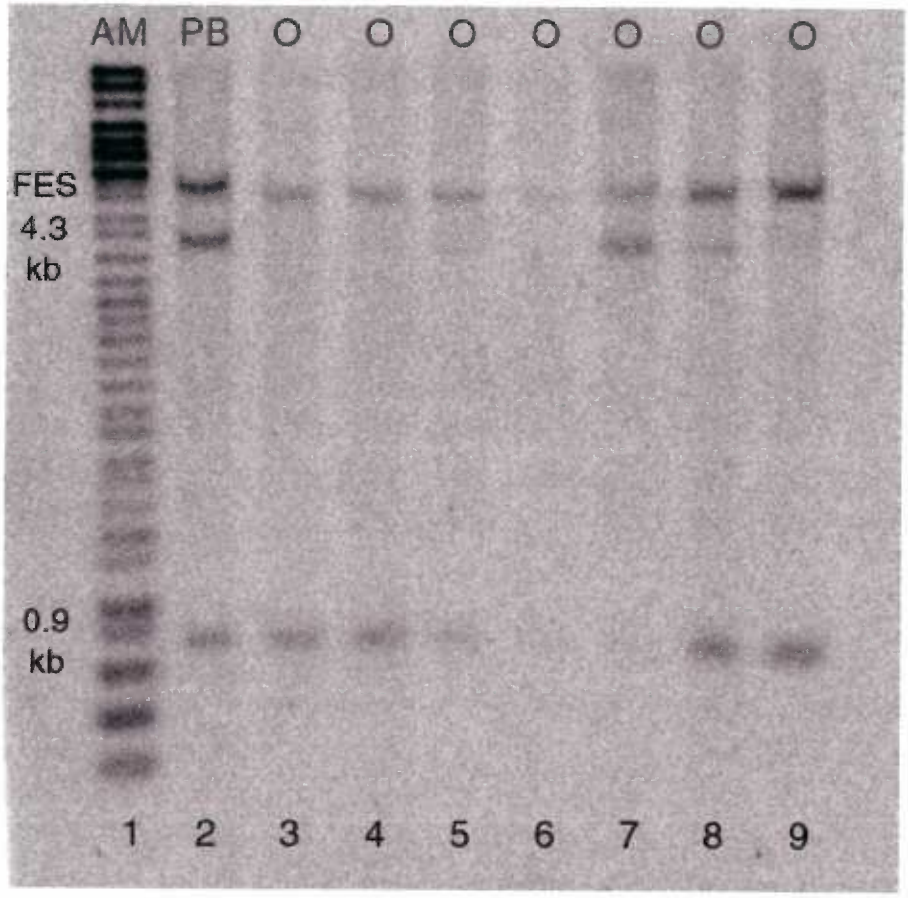

Figure 5.2: *SNRPN* Methylation Patterns in Frozen Tumor Samples From Pediatric GCTs DNA was extracted from flash frozen tumor samples, digested with *Xba*I and *Not*I, and probed to detect the methylation pattern at the 5'UTR of *SNRPN*. Normal somatic cells yield a maternally derived, methylated 4.3 kb band and a paternally derived, unmethylated 0.9 kb band. An additional probe for *FES* (15q26) was included as a control for gel loading. Notice the difference in intensity between the 4.3 kb and 0.9 kb bands in lanes 4 and 8. **AM**, analytical marker (Gibco/BRL); **PB**, normal peripheral blood; **O**, ovary

Summary and Conclusions

Cytogenetics

This study represents the largest survey of the cytgenetics of pediatric GCTs done to date and has identified the recurrent, non-random chromosome abnormalities present in these tumors. These abnormalities were then correlated with patient sex, age, tumor location and histology that stratified patients into distinct groups. The currently recognized groupings of malignant GCTs in children were based on patient age, sex, and tumor location and defined the following categories: (1) the GCTs of young children; (2) the GCTs of the adolescent gonad; and (3) the extragonadal GCTs of the adolescent male (Castleberry et al. 1997). The data from this study provide the first evidence that the GCTs of the adolescent gonad can be divided into those of the ovary and those of the testis and may represent two distinct entities. Although there was some indication, based on both clinical behavior and presentation, that the GCTs of young children could also be subdivided on the basis of patient sex, there was not sufficient data among the malignant tumors from this group to make a firm statement in that regard.

The cytogenetic groupings appear to reflect an underlying biology of germ cells that is influenced by patient age and sex. The similarity of the chromosome abnormalities in the ovarian tumors of girls of all ages suggests the initiating germ cells were at a similar stage of development. This is a reasonable conclusion given that the germ cells of a newborn or 16 year old girl are arrested in the same stage of meiosis I. The differences in the numbers and the histologic types between the ovarian tumor of young girls versus those from adolescents may be indicative of the influence of a changing hormonal environment brought about by the onset of puberty. The sacral/retroperitoneal tumors from girls and boys under the age of four can be postulated to also share a common germ cell origin, namely a primordial germ cell that fails to migrate to the genital ridge. Differences seen between the sexes might again be explained by exposure to different hormonal environments.

Cytogenetic abnormalities common to more than one subgroup, such as loss of 1p and whole chromosome gains of chromosome 3 and 21, may be indicative of similar pathogenetic mechanisms operating in these tumors that are then influenced by other factors. The whole chromosome gains are of particular interest because many of the tumors in our series were characterized by simple abnormalities including gains of whole chromosomes. The non-random gain of whole chromosomes is a common theme in cancer cytogenetics with many tumor types demonstrating this finding. Recent work in yeast may shed light on the process by which a chromosome is gained non-randomly. Deletions of certain genes in yeast result in the non-random gain of single chromosomes. When the chromosomes are analyzed, often a paralog to the deleted gene is present on the gained chromosome (S. Friend, unpublished). Common non-random chromosome gains such as +3 or +21 in these tumors could reflect deletions of genes that play a more general role in germ cell tumorigenesis.

Chromosome 1p FISH

During the course of the study, it became apparent that abnormalities of the short arm of chromosome 1 leading to a loss of 1p material and over-representation of 1q material were common in pediatric GCT. Furthermore, such abnormalities were found in multiple subgroups, suggesting a more general role in the pathogenesis of these tumors. At the same time, reports in the literature suggested the loss of 1p was more common than previously thought and could possibly be associated with malignancy in this tumor type (Stock et al. 1994; Jenderny et al. 1995; Jenderny et al. 1996; Perlman et al. 1996). We tested the hypothesis that 1p loss was more common in malignant pediatric GCTs than benign pediatric GCTs in our data. We found loss of 1p to be more common than initially demonstrated by G-banding. We also found a significant association of 1p loss with malignancy.

The gene(s) responsible for the resultant malignant phenotype have not been identified. Loss of 1p is a common finding among many tumors. The region deleted in pediatric GCT overlaps the region deleted in neuroblastoma, and the two tumor types share some features in

common when neuronal elements are present in the GCT, including amplification of *MYCN* and neuron specific enolase production (Ishiwata et al. 1991). This suggests that the same gene(s) may be involved in both tumor types. The *CDC2L* locus, which encodes PITSLRE kinase, a homolog of $p34^{cdc2}$ thought to be involved in apoptosis, is located 10 kb from D1Z2, the locus we screened for in our study. Every tumor in our study that had a deletion of D1Z2 also had a deletion *CDC2L*. Alterations and mutations of *CDC2L* have been identified in neuroblastoma and melanoma (Lahti et al. 1994; Nelson et al. 1999). While loss of PITSLRE kinase may play a role in pediatric GCTs, it is unlikely that it is the only or primary target of the deletion. First, the cytogenetically visible deletions are large, often encompassing the entire short arm of chromosome 1. Secondly, recent work demonstrated that the smallest region of overlap (SRO) for neuroblastoma encompasses a 5 cM region from D1S508 to D1S244, excluding *CDC2L* (Abel et al. 1999). This SRO also excludes p73, a homolog of p53. The clarification of the role of PITSLRE kinase in pediatric GCTs requires more detailed mapping of the 1p deletions by FISH with cytogenetically ordered probes for the region (now becoming available from the Cancer Chromosome Anatomy Project) to determine the SRO for pediatric GCTs. It would also necessitate looking for mutations in the *CDC2L* gene cluster in malignant pediatric GCTs that do not show a deletion for the distal region of 1p.

Chromosome 12p FISH

Our G-band studies of pediatric GCT failed to demonstrate a high incidence of an isochromosome for the short arm of chromosome 12 in tumors other than those from adolescent males. However, because 12p amplification can be present in other chromosome rearrangements, and the possibility that the 12p amplified clone was not dividing and therefore not analyzed in these tumors, we looked for 12p amplification by FISH in the tumors of both sexes. Among the males, we saw no 12p amplification that was not previously identified by G-bands, and the tumors with 12p amplification were all from patients who were

over the age of 9. By combining our data with that in the literature, we concluded that 12p was amplified more commonly, and may be exclusively amplified, in tumors from boys over the age of 9. This was a significant result and provides further support for an etiological difference between the tumors of younger boys and those from adolescents and adults.

Among the females, we failed to identify amplification of chromosome 12p in the vast majority of the tumors, including among the dysgerminomas. This is in conflict with the data in the literature that suggest that at least among dysgerminomas of the ovary, amplification of 12p is common (Atkin and Baker 1987; Speleman et al. 1990; Rodriguez et al. 1995; Baker et al. 1998; Riopel et al. 1998; van Echten et al. 1998). This may represent a sample bias in our data as we had few dysgerminomas, and most of them were karyotypically normal. This suggests in turn that we may have a confounding factor of selective outgrowth of normal stroma. To address this problem, we would need to obtain archival, uncultured material for our dysgerminomas and then perform FISH and/or CGH to look for 12p amplification.

The selective advantage of 12p amplification in GCTs is a subject of intense investigation. 12p11.2-12.1 is the smallest common region of amplification among adult testicular GCTs (Mostert et al. 1996). The gene for cyclin D2 is located in this region, has been shown to be over-expressed in adult testicular GCTs, and is a candidate for an initial transforming event of germ cells (Murty and Chaganti 1998). However, the relationship between 12p amplification and patient age has not been explained. The association suggests that there may be selection for an androgen responsive gene on 12p. Recent work on fine mapping of 12p amplification in adult testicular GCTs using cDNA microarrays has identified 14 clones located on 12p (V. Bourdon, unpublished). These include novel expressed sequence-tagged sites that were obtained from testicular or germ cell libraries. Alternatively, the association of 12p amplification and age may be indicative of a different precursor giving rise to the GCTs in young boys as compared to adolescent males. The finding of 12p amplification in the extragonadal tumors of males over the age of 9 (1 case from our series

and 2 in the literature) argues in favor of an androgen responsive gene, as the precursor giving rise to tumors in extragonadal locations is likely to be at a different developmental stage than that giving rise to tumors in the testis. An experiment by which the gene expression of male germ cells exposed to androgen is compared to germ cells not exposed to androgen, and the data is analyzed for genes that map to chromosome 12 would address this question. One would be looking for upregulated genes mapping to 12p11.2-12.2 and downregulated genes mapping to 12q. This type of experiment could be done in mouse, examining genes mapping to the syntenic regions of the mouse genome. Similar work has been done to look at *MYCC* amplification (T. Reid, unpublished).

SNRPN Methylation and Timing of Mutation Events

Understanding the timing of the initiating events leading to the development of GCTs and the mechanisms leading to tumor formation is necessary in order to design preventive strategies. The current opinion in the literature is that the initial events leading to tumor development occur early, sometime during fetal life (Castleberry et al. 1997). We studied the methylation pattern of the 5' untranslated region the imprinted gene SNRPN to establish the "imprinting stage" of the PGC precursor of the GCT. Because we can predict what the methylation pattern should be at this locus for a tumor in a given location, and we know about the timing of the events leading to changes in this pattern during development, we can tentatively establish a time reference for the initiating events leading to tumor formation. The data suggest that the events leading to tumorigenesis are acting upon PCGs as early as 24 days of fetal life (the time at which PCGs start migrating to the genital ridge). In this context, events leading to the presentation of congenital sacral GCTs are possibly directly oncogenic. However, tumors arising later in childhood could originate from a PGC that sustained a predisposing mutation in the early time frame. A second, oncogenic event would then be necessary for tumor development. The presence of constitutional chromosome abnormalities that lead to dysgenetic gonads may be one such predisposing hit.

The data from the *SNRPN* studies strongly supports a similar model of imprint erasure and resetting between mice and humans for all locations except for the sacrum. This is not because of a lack of concordance between expected and observed results. Rather it stems from the lack of discriminatory power of the expected result to distinguish a germ cell origin from a somatic cell origin of tumors in this region. The PGC origin of GCTs has been questioned in extragonadal tumors (Castleberry et al. 1997). The methylation data in the anteriorly located extragonadal tumors suggests that a subset of GCTs from these locations is PGC in origin, but the remainder have a somatic cell origin. To tease apart the origin of GCTs from the sacrum, the logical step is to examine specific markers of germ cells versus pluripotent embryonic stem cells. Possible currently known markers include Oct-3/4 expression (Pesce et al., 1998) and neural crest specific markers, such as p75 antigen (Morrison et al. 1999). The two drawbacks to studying Oct-3/4 expression are: (1) Oct-4 expression may disappear as germ cell tumors undergo differentiation along embryonic or extraembryonic lineages meaning that lack of Oct-4 expression does not rule out germ cell origin; and (2) Embryonic stem (ES) cells express Oct-4, so expression does not unequivocally support germ cell origin. Work is ongoing to determine differential gene expression for embryonic germ cells versus embryonic stem cells in the mouse. Once this work is reported, other more appropriate markers might be available.

The issue of timing needs to be addressed for the GCTs of the testis. Because the methylation pattern at *SNRPN* is the same for germ cells that are premeiotic and those that have entered meiosis, determining a finer level of timing is impossible by *SNRPN* methylation patterns alone. To further define the time period of interest, matched pairs of tumor and patient blood would need to be examined for evidence of crossing over, as has been done for benign ovarian teratomas. Limited work in this respect suggests that testicular GCTs arise from a premeiotic germ cell (Hoffner et al. 1994).

Future Directions

Cytogenetic analysis of pediatric GCTs needs to continue. Although the karyotypes of over 100 tumors have been published to date, there are still too few tumors of any given histologic type to examine possible differences in chromosome abnormalities in conjunction with patient sex, age, and tumor location. A particular focus should be on the malignant GCTs of children under 4 to determine if there is a difference in the patterns of chromosome abnormalities associated with patient sex. The cytogenetic results need to be supplemented with ongoing FISH for 1p loss, to allow further mapping of the SRO for pediatric GCTs, and FISH for 12p amplification to continue to investigate the incidence of 12p abnormalities and their possible significance in female GCTs. Tissue microarrays, a method by which several hundred tumors can be screened at once may be useful for a retrospective application of the FISH studies. Once the SRO on 1p is more defined, using microarrays to further map the deletions would be appropriate. In addition, CGH and M-FISH/SKY needs to be done on cytogenetically characterized pediatric GCTs to enhance the current database of abnormalities.

Very little data exists for the gene expression profiles for pediatric GCTs. A microarray approach that targeted known oncogenes and tumor suppressors and their pathways would be useful. Adult testicular GCTs express high levels of wild-type p53, explaining their sensitivity to chemotheraputic agents (Murty and Chaganti 1998). However, the ability of these same cells to continue to divide suggests that other downstream targets of p53 are inactive. A similar situation might exist in the pediatric GCTs. As the role of 12p amplification is better defined, the gene(s) involved could also be included in such experiments to further delineate the differences between adult and pediatric tumors.

Beyond exploring the basic biology of these tumors, the results gained from such studies need to be correlated with the clinical course of the tumors. Studies need to be done to determine whether the presence of 1p loss in malignant pediatric GCT is associated with poor prognosis. Another question to be addressed is whether the spectrum of chromosome

12 abnormalities found in GCTs correlate with treatment response. As more tumors are cytogenetically characterized, the clinical outcomes of the patients need to be further studied with respect to cytogenetic patterns identified in the tumors. Initial work in this respect has been done by the Children's Cancer Group for two studies, CCG-8891 and CCG-8892, investigating the outcomes of different treatment regimens for low risk and high risk tumors, respectively. Thus far, these studies have demonstrated that surgery alone is effective for the management of localized immature teratomas and stage I malignant GCTs from the testis and might be sufficient for stage I ovarian GCTs and extragonadal tumors (Cushing et al., 1999). The use of high-dose cisplatin, etoposide, and bleomycin after surgery has improved event-free survival from 50% to 90% among high risk tumors (Giller et al., 1998; Cushing et al. 1998). However, there are issues of toxicity, especially non-reversible ototoxicity, associated with this regimen. These studies are still in the five-year follow-up period, so no analysis of chromosome abnormalities with respect to patient outcome has been conducted yet. The goal of such studies would be to identify specific chromosomal changes or patterns of changes that might serve as an independent assessment of risk and eventually lead to targetted therapies with little or no side effects.

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