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# Utilization of FISH for Constitutional and Acquired Chromosomal Abnormalities for Diagnostic and Prognostic Purposes

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Thesis submitted to the
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at West Virginia University

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Master of Science in
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# ABSTRACT

## Utilization of FISH for Constitutional and Acquired Chromosomal Abnormalities for Diagnostic and Prognostic Purposes

Amy L. Shackelford

Fluorescence in-situ hybridization (FISH) is a useful molecular cytogenetics technique for counting chromosomes and identifying specific chromosomal sequences of interest. FISH probe targets include centromeres, single loci, subtelomeres, and telomeres, using DNA or peptide nucleic acid (PNA) probes. FISH probes were used to determine chromosome number, copy loss or gain, and signal size in four studies involving acquired chromosomal changes in malignancy, mosaicism, and aneuploidy in pre- and postnatal constitutional abnormalities.

In the first series of experiments, subtelomere probes for 5p and 5q were used to evaluate paraffin embedded patient samples for a 5q deletion and compare that to pathological and clinical characteristics of small cell lung carcinoma (SCLC). The correlation between del(5q) and spindle cell morphology was found to be significant ($p<0.025$), but of unknown relevance. In the second study, FISH was used in conjunction with microarray analysis to define the karyotype of a patient with a Pallister-Killian (PKS) phenotype, a tissue-limited mosaic condition. Her karyotype was determined to be 46,XX,dup(12)(p11.2p13.2),trp(12) (p13.2pter)[5]/46,XX[15], with the abnormal cell line remaining at 25% from 6 to 19 months of age. PNA telomere length studies were performed on this same patient to determine if there was a difference between the normal and abnormal cells lines, shorter telomeres explaining loss of abnormal cells, however, no difference was found. In the third series of studies, samples from pregnancy losses that failed to grow in culture were investigated using FISH. Two of the five culture failure samples identified a mosaic trisomy 9 female and a mosaic tetraploid female using FISH probes in interphase cells. In the fourth and final study, PNA FISH probes were used to assess the difference between telomere lengths in newborns with trisomy 21 and normal chromosomes. Telomere lengths in cells with trisomy 21 were significantly shorter than those with normal chromosomes in both metaphase ($p<0.05$) and interphase ($p<0.01$) cells. Cell senescence with shortened telomeres, would correlate with shorter lifespan of individuals with trisomy 21.

Overall, FISH is an important tool that can enhance the diagnostic capabilities of conventional cytogenetics testing, particularly when used in conjunction with traditional karyotyping. FISH offers a more rapid alternative when turn around times are critical and allows for more specific resulting, particularly in cases where an abnormality of interest is unable to be visualized within a karyotype, specimens are nonliving, or the study of interphase cells is necessary.

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## Literature Review

Karl Wilhelm von Nägeli first described "transitory cytoblasts" in plant nuclei and their behavior at cell division in *Zeitschrift für wissenschaftliche Botanik* in 1844.[1] In 1880, Walther Flemming published work investigating chromosome morphology and the movement of chromosomes during mitosis, including detailed sketches of the process.[2,3] Leading from Flemming's work, Wilhelm von Waldeyer-Hartz coined the term "chromosome" in 1888, from *chromos*- Greek for color and *soma*- Greek for body, due to the ability of chromosomes to be stained for visualization.[4]

Approximately 40 years later, the field of human cytogenetics found its beginnings with the declaration that normal human cells possess 48 chromosomes. In the early 1950s, the accidental addition of a hypotonic salt solution to a human mitotic cell suspension prior to fixation and slide preparation caused the cells to swell, allowing the chromosomes to spread and be visualized individually.[5] In 1956, Tijo and Levan, utilizing the hypotonic technique, initially established the human chromosome count to be 46, which Ford and Hamerton confirmed independently within the same year.[6,7] Cell culturing and harvesting techniques continued to be improved, notably with the addition of phytohemagglutinin to stimulate cell cultures and of colchicine to arrest cells in metaphase. Over time, chromosomes were paired and sorted into groups (A through G) by size and centromeric position, or karyotyped.

Karyotyping rapidly changed the landscape of human genetics as relationships were demonstrated between chromosomal aneuploidies and abnormal phenotypes, specifically trisomy 21 and Down syndrome, X and Turner syndrome, XXY and Klinefelter syndrome, and an association was seen between miscarriages and aneuploidy.[8] Chronic myelogenous leukemia (CML) was characterized by the Philadelphia chromosome, a G group chromosome with a deletion, in 1960.[9] Though incorrect, this set the stage for recognition of chromosomal abnormalities as causal to disease. The development of banding and staining methods in the late 1960s helped to clarify what was seen in solid-stained karyotypes and allowed researchers to study structural chromosome abnormalities such as translocations, inversions, deletions, and duplications and their relationship to abnormal phenotypes.[10,11]

Additionally, in the late 1960s, molecular hybridization was used to identify the positions of DNA sequences *in situ*, or in their natural loci within chromosomes. Researchers Joseph Gall and Mary Lou Pardue hybridized tritium-labeled copies of an RNA sequence to complementary DNA sequences in the nuclei of *Xenopus* toad oocytes and visualized the hybrids using autoradiography.[12] Radiographic methods based on their research were utilized into the 1980s when fluorescent techniques were developed and fell into common usage. Radioactive substances are inherently unstable, are hazardous, and require special disposal methods. In addition, it took a relatively long time to measure radioactive signals emitted by the probes. The changeover to fluorescent techniques overcame most of these obstacles while adding the ability to simultaneously detect multiple targets and analyze probe signals quantitatively.[13]

The first fluorescent in situ detection took place in 1980, via the utilization of an RNA probe that was directly labeled at the 3' end with a fluorophore.[14] Fluorescence in situ hybridization (FISH) methodology was developed by Pinkel and associates in 1986 using fluorescent-labeled probes to detect specific DNA sequences.[4,15] Simultaneous two-color and three-color FISH methodologies followed within the decade.[13] Further advances in FISH would allow researchers and diagnosticians to increase the resolution of the traditional karyotype and to visualize specific loci on chromosomes within interphase cells, non-living/dividing cells, and tissue on paraffin-embedded slides.

Genome mapping allowed for the widespread identification of gene loci, resulting in the ability to characterize abnormal chromosomes by FISH to identify affected genes. One such example was the characterization of genes involved in the breakpoints common to the inv(16) seen in acute myelogenous leukemia (AML) patients. FISH was crucial to the identification of the MYH (smooth muscle myosin heavy chain 11) and CBFB (β-subunit of core-binding factor) genes that result in transformation to the AML phenotype when fused.[8,16] These types of studies paved the way for the ability to associate specific chromosomal abnormalities previously not visible in the karyotype with abnormal phenotypes. The clinical utilization of FISH studies to determine which genes are involved in chromosomal abnormalities assists in determining an explanation, via the identification of gene(s) involved combined with the knowledge of gene function, for the abnormal phenotype that results.

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## Introduction

Fluorescence in-situ hybridization (FISH) is a molecular cytogenetics technique developed in the mid-1980s for counting chromosomes and identifying specific chromosomal sequences of interest.[1] FISH involves the tagging of specific chromosome sequences via the hybridization of a fluorescent-tagged complementary DNA probe to the sequence of interest on a selected chromosome. This is accomplished by warming slides with tissues or cells affixed so that the DNA in the cells denatures, or opens up, allowing a complementary sequence (probe) containing a fluorescent tag to be hybridized to the sequence of interest.[2] These fluorescent tags can then be visualized using a fluorescent microscope and the signals showing interpreted to determine the presence (signal present) or absence (no signal seen) of the sequence/gene being studied. There are two major types of FISH probes, repetitive or single copy, or chromosome enumeration and locus specific respectively. Chromosome enumeration probes typically hybridize to the centromere, which consists of repetitive sequences unique to a specific chromosome, and are used to determine the number of copies of a chromosome present. Locus specific probes hybridize to a gene, or genes, of interest and can be used to determine deletions, amplifications, or translocations. Subtelomere probes are used to evaluate the presence of the subtelomeric regions of specific chromosomes while peptide nucleic acid (PNA) probes hybridize to the telomeric regions, which are repetitive DNA sequences, on chromosomes to allow for the measurement of telomere lengths.

Some advantages to FISH are the ability to utilize FISH on nonliving cells (paraffin-embedded tissue), previously harvested cell cultures, non-dividing or interphase cells, its specificity to certain genes, faster turn around times, and the ability to visualize abnormalities that cannot be seen with traditional karyotyping. Disadvantages include hybridization artifacts, background fluorescence, and an inability to visualize hybridization results within the context of a metaphase cell. Overall, FISH is a successful technique when utilized for prognostic information in oncology and in constitutional cases, both pre- and post-natal, where specific translocations, rearrangements, deletions, or amplifications are a concern, and also to determine aneuploidy.

In the following projects, FISH was utilized to evaluate copy numbers of chromosomes and/or locus specific regions for acquired changes in oncology and

constitutional abnormalities in prenatal and postnatal cases. In Chapter 1, subtelomere probes for 5p and 5q were used to determine 5q deletions in tissue samples of patients with SCLC with specific pathological and clinical characteristics of the disease. In Chapter 2, enumeration and locus specific probes were used to determine the number of copies of these regions that were present on chromosome 12p in peripheral blood from a patient who was mosaic for a chromosome abnormality. PNA probes were used to compare telomere lengths in the abnormal and normal cells. In Chapter 3, a variety of centromere enumeration probes and locus specific probes were utilized in the determination of aneuploidy in products of conception samples that either failed to grow in culture or had a 46,XX karyotype. In Chapter 4, PNA probes were used to evaluate telomere lengths in newborns with trisomy 21 to compare with telomere lengths in newborns with normal chromosomes.

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# CHAPTER 1: Pathological and Clinical Characteristics of Small Cell Lung Carcinoma Associated with 5q Deletion

## Introduction

Small cell lung carcinoma (SCLC) is an aggressive neuroendocrine carcinoma that exhibits rapid growth, early metastasis, and unique sensitivity to radiation and chemotherapy. Annually, SCLC accounts for approximately 15% of new lung cancer diagnoses and as many as 25% of lung cancer deaths in the United States.[1] SCLC is the most aggressive subtype of lung cancer, with an overall 5-year survival rate of about 5%.[2] Cigarette smoking has been implicated in approximately 95% of all SCLC cases.[1]

Carcinogenesis is the result of altered oncogenes and tumor suppressor genes, via activation of the former and inactivation of the latter. The tumor cells achieve growth advantage, uncontrolled proliferation, and metastatic behavior through the disruption of key cell-cycle regulators and signal transduction cascades.[3] Respiratory epithelial cells require multiple genetic alterations to become cancerous. Relevant chromosomal abnormalities involve deletions of 3p, 4p, 5q, 16q, 13q, and 17p.[4] Del(5q) is found to occur in >60% of SCLC cases, making it one of the most common cytogenetic findings, and thus worthy of further investigation.[5,6,7,8] Deleted regions on 5q have been correlated with SCLC, specifically 5q11-13 containing the hMSH3 mismatch repair gene, 5q21 which includes the APC and MCC tumor-suppressor genes, the EGR1 locus at 5q31, and the CSF1R/PDGFRB region at 5q33-q35.[7,8,9,10,11] The purpose of this study was to assess the relationship between del(5q) and the clinical and histological characteristics of SCLC.

## Materials and Methods

Institutional review board approval was obtained for this retrospective study. Thirty-six cases of SCLC from 1998 to 2005 were selected and assessed by two pathologists for histological parameters including mitotic count, cell morphology, spindle versus ovoid cells, degree of necrosis and fibrosis, neuroendocrine morphology, and mucin production. For each case, 3-mm thick tissue was placed on glass microscope slides by Histology. Pathologists marked the abnormal areas on one hematoxylin and eosin (H&E) stained slide per case. Unstained slides were then etched to reflect the H&E

markings. To prepare for fluorescence in situ hybridization (FISH) analysis, one slide per case was deparaffinized using SkipDewax (Insitus Biotechnologies) at 80°C for 20 min, followed by two 3-min distilled water washes. Slides were then placed in a pepsin solution (25 mg pepsin, 49.5 mL distilled water, 0.5 mL 1N HCl, pH 2.0) for 1 hour at 37°C, followed by two 5-min washes in 2x sodium chloride/sodium citrate (SSC, pH 7.0). The slides were dried on a 50°C slide warmer for 5 min.

Subtelomere probes for 5p and 5q (Vysis TotelVysion cocktail #5; Abbott Molecular) were diluted (2:50 µL) in tDenHyb (Insitus Biotechnologies). The probe mixture was applied to the previously etched area of the tissue in an amount relative to the size of the sample and sealed under a cover glass using rubber cement, typically 5 µL of probe mixture under an 18-mm round glass. Slides were placed in a ThermoBrite slide warmer (Abbott Molecular) at 90°C for 3 min to denature the DNA. The slides were then transferred to sealed, humid slide boxes in a 37°C water bath to hybridize for 16 to 20 hours.

After hybridization, the rubber cement and cover glasses were removed and the slides were washed in 2xSSC/0.3% NP-40 (pH 7.0) at 72°C for 2.5 min and then allowed to dry in the dark for 10 min. Twenty mL of 4',6-diamidino-2-phenylindole (DAPI) counterstain was then applied and each slide was sealed with a 20x50 mm cover glass.

A Leica epi-fluorescent microscope equipped with DAPI single band-pass and red/green dual band-pass filters was used for signal enumeration of the red and green signals from the subtelomere probes. Up to 100 non-overlapping cells were scored by two individuals. Red signals indicated the 5q telomeric region and green signals indicated the 5p telomere region. A red-to-green ratio less than 0.80 indicated a 5q terminal deletion.

**Results**

Of the 36 cases studied, 33 hybridized successfully for FISH analysis. Of these 33 cases, 15 were identified as having the 5q terminal deletion (Figure 1). Of these 15, eight were male and seven female with an overall mean age of 71. Five of these patients were diagnosed incidentally, while four presented with shortness of breath, and four with cough. This group had a mean smoking history of 61 pack-years, defined as (packs

smoked per day) x (years smoking). Eight were at level 4 staging. Mean survival for this group was 20 months.

Of the 18 patients without the 5q terminal deletion, nine were male and nine female, with a mean age of 59. Seven presented with shortness of breath, the only common symptom at diagnosis. This group had a mean smoking history of 44 pack-years. Twelve were at level 4 staging. Mean survival for this group was 21 months. Six of the cases that did not have the 5q terminal deletion were reevaluated for interstitial deletion using the EGR1 FISH probe for the 5q31 locus, which was also not deleted.

Eleven patients with del(5q) had spindle cell morphology compared with six in the group without a 5q deletion. This difference in spindle cell morphology, as defined by the presence in a minimum of 50% of tumor cells, was significant ($p<0.025$). None of the other histological parameters studied showed differences between the del(5q) group and those without the deletion.

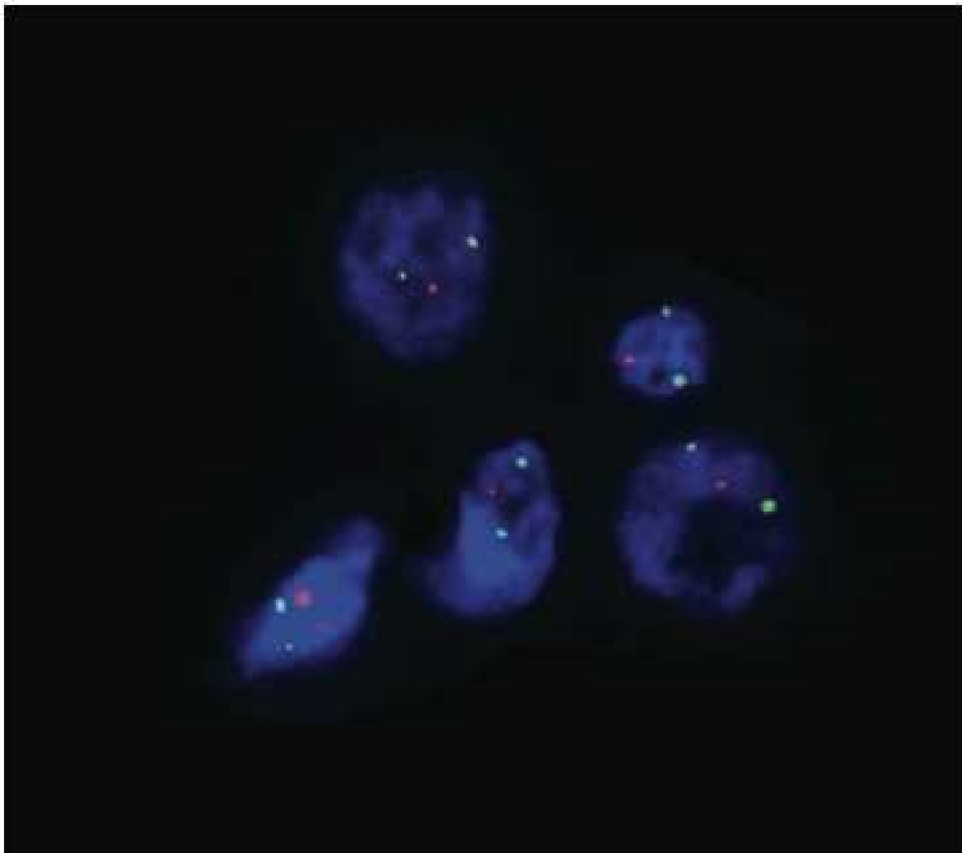

Figure 1: FISH picture showing SCLC tumor cells with 2 green signals and only 1 red signal signifying the del(5q). The tumor cell on the lower left shows 2 green and 2 red signals, absent the del (5q).

**Discussion**

Del(5q) is found in >60% of SCLC cases and several regions on 5q have been correlated with SCLC, such as 5q11-13 which includes the hMSH3 mismatch repair gene, 5q21 which includes the APC and MCC tumor-suppressor genes, the EGR1 locus at 5q31, and the CSF1R/PDGFRB region at 5q33-q35.[7,8,9,10,11] It is therefore relevant to explore the relationship between histological findings in SCLC neoplasia and the 5q deletion. Previous studies have associated advanced disease stage and poor survival rates with del(5q), which this study did not confirm.[7,12,13] Current literature mentions the association between del(5q) and SCLC, but does not reflect attempts to assess the meaning of such an association.

Of the 33 cases studied, there were no significant differences between the del(5q) cohort and those without the deletion with regard to sex, average post-diagnosis survival time (20 and 21 months, respectively), staging level at diagnosis (50-60% of both groups were at level IV), or mucin production. The del(5q) group was older on average, had a higher number of pack-years smoked, and had more varied symptoms upon diagnosis. The larger number of pack-years in the older group is likely a factor explained by age. It follows that the increase in 5q deletions seen in the older group may be the result of the longer duration of smoking and increased exposure to the carcinogens present in cigarette smoke. In addition, it may be possible that the del(5q) occurs later in SCLC progression in some patients. The patients without the 5q deletion were younger and all were symptomatic at the time of diagnosis in contrast to the del(5q) group, a third of which were diagnosed incidentally. Despite having a similar survival time after diagnosis, this may reflect the aggressiveness of the cancer progression in the cohort without a deletion. It follows that the del(5q) may indicate less aggressive tumor progression characterized by later onset and fewer symptoms.

Histologically, spindle cell morphology, as defined by its presence in >50% of tumor cells, occurred twice as frequently in the del(5q) cohort, which was statistically significant ($p<0.025$). There were no significant differences between the groups with regard to mitotic count, degrees of necrosis or fibrosis, mucin production, or neuroendocrine morphology. The significance of variation in spindle cell morphology with regard to the presence/absence of del(5q) is unknown at this time. The use of a

subtelomere probe to assess del(5q) leaves room for uncertainty in that it will not detect interstitial 5q deletions. In addition, given the small sample size and that all testing was performed on biopsies that are only representative of a portion of the tumor, further studies would be necessary to determine if there is any true relationship between the variables investigated and del(5q).

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# CHAPTER 2: Persistent Mosaicism for 12p Duplication/Triplication Chromosome Structural Abnormality in Peripheral Blood

## Introduction

Patients with trisomy 12p typically present with severe mental retardation, seizures, low-set ears, and characteristic facial dysmorphology including flatness of the face, small nose with broad bridge, anteverted nares, inner epicanthal folds, long philtrum, everted lower lip, and high forehead. The extra copy is due to an intrachromosomal duplication or an extra copy on a derivative chromosome. Patients with tetrasomy 12p, or Pallister-Killian syndrome (PKS), additionally present with sparse temporal hair, eyebrows, and eyelashes, prominent forehead, a cupid-bow shaped mouth, and large ears. A hallmark of PKS is tissue-limited mosaicism, with few, if any, abnormal cells found in peripheral blood lymphocyte metaphases in the newborn. Another characteristic of tetrasomy 12p is loss of the abnormal cell line in peripheral blood and skin fibroblasts as the patient ages or over time in serial-passaged cultured fibroblasts.[1,2]

Approximately 26 individuals with nonmosaic structural 12p duplications or triplications have been reported in the literature with a minimum critical region of 12p13.31.[3] Only about 24 cases with mosaicism for a structural abnormality of an autosome have been reported in the literature.[4] The patient presented in this chapter has mosaicism in peripheral blood for a derivative chromosome involving duplication and triplication of 12p. The purpose of this study was to determine which portions of the 12p the patient had in multiple copies, the parental origin of the extra 12p material, and possibilities for why the patient has the PKS phenotype, but is failing to lose the abnormal cell line in her peripheral blood over time.

## Clinical Report

The patient was delivered at 38 weeks gestation to a 19-year-old mother. The pregnancy was complicated by gestational diabetes and polyhydramnios. At birth, the infant presented with an anal fistula, hypertension, mild hypotonia, ventricular septal defect (VSD), and intraventricular hemorrhage and was hospitalized for 3½ weeks due to

breathing difficulties. At 5 months of age, the patient was able to roll over and sit with support and had exotropia. She was seen by genetics at 6 months of age and was found to be dysmorphic and hypotonic with significant developmental delays. At ten months, she was unable to crawl or sit without support. Her height and weight were in the 50th percentile, with head circumference at the 90th percentile. At 19 months, the patient had a broad, high forehead, bitemporal balding, small posteriorly rotated ears, global developmental delays, and mild hypotonia. She could sit but not pull to a stand. She was asymptomatic for VSD, had eye surgery to remove chalazia, and was receiving physical, speech, and vision therapies as well as seeing a developmental specialist.

## Materials and Methods

Peripheral blood, obtained from the patient and her parents, was processed using routine cytogenetic procedures to obtain a karyotype. One mL of peripheral blood was incubated in 0.9 mL of PBMax PHA stimulated media (Life Technologies) for 72 hours. Eighty µL of Colcemid was added and the samples incubated for 20 min at 37°C. Samples were then spun at 1,500 RPM for 5 min and the supernatant aspirated. Ten mL of KCl were added and the samples incubated at 37°C for 10 min. Two mL of Carnoy's fixative (3:1 methanol to glacial acetic acid) was added and the sample spun again at 1,500 RPM for 5 min, followed by three additional washes with fix. Slides were dropped and G-banded following standard procedure. Twenty metaphase cells per parent and 100 metaphase cells on the patient were analyzed for the presence of an abnormal 12p.

FISH was performed on the patient's peripheral blood metaphases using Vysis probes (Abbott Molecular) for centromere 12, TEL (12p13), and subtelomere 12p. Slides were dropped and placed in 2xSSC for 30 min, followed by 5 min in pepsin/HCl (25 mg pepsin, 0.5 mL 1N HCl, 49.5 mL distilled water), 1 min in 2xSSC wash, 5 min in 1% formaldehyde solution (1 mL 37% formaldehyde, 1.9 mL $MgCl_2$, 39 mL 1xPBS), and an addition 1 min in 2xSSC wash. Slides were then dehydrated in a 70%, 85%, and 95% ethanol series at 2 min each and air dried. All probes were diluted (2:50 µL) in *c*DenHyb (InSitus Biotechnologies) and 20 µL applied to the slides under a 24x50 mm glass coverslip, which was then sealed with rubber cement. Slides were placed in a ThermoBrite slide warmer (Abbott Molecular) at 90°C for 3 min to denature the DNA.

The slides were then placed in sealed, humid slide boxes in a 37°C water bath to hybridize for 16 to 20 hours.

After hybridization, the rubber cement and cover glasses were removed and the slides washed first in 0.4xSSC/0.3%NP-40 for 2 min at 73°C and then in 2xSSC/0.1%NP-40 for 1 min at room temperature. Slides were allowed to dry in the dark and then 20µL of DAPI counterstain was applied and covered with a 24x50 mm coverslip. One hundred cells were scored on each probe to determine the percentages of normal and abnormal cells.

Metaphase cell telomere lengths were compared between the normal and abnormal cell lines using the Telomere Peptide Nucleic Acid (PNA) FISH FITC kit from Dako (DakoCytomation). PNA probes for all telomeres were utilized in conjunction with the Vysis 12 centromere probe and all probes were hybridized simultaneously according to Dako's PNA instructions. Slides were dropped and placed in a tris-buffered saline (TBS, pH 7.5) pre-wash for 2 min, followed by 3.7% formaldehyde solution (5 mL 37% formaldehyde in 50 mL TBS) for 2 min, two TBS washes at 5 min each, a pre-treatment solution provided by Dako (2000 x concentrated proteinase K diluted 1:2000 in TBS) for 10 min, and two additional TBS washes at 5 min each. Finally, slides were dehydrated in a 70%, 85%, and 95% ethanol series at 2 min each and tipped vertically to dry. Twenty µL of Dako PNA probe previously mixed with 3 µL of Vysis CEP 12 probe was applied to the slides under a 24x50 glass coverslip. The slides were then placed in a ThermoBrite slide warmer at 80°C for 5 min and afterward kept in the dark at room temperature for 30 min.

After hybridization, the slides were immersed in Dako rinse solution (diluted 1:50 in distilled water) for 1 min to remove the coverslips. The slides were then washed in the Dako wash solution (diluted 1:50 in distilled water) for 5 min at 65°C, followed by the 70%, 85%, and 95% ethanol series at 2 min each, and allowed to dry flat for 5 min in the dark at room temperature. Twenty µL of DAPI counterstain was applied and covered with a 24x50 coverglass. Telomere lengths were measured in 30 cells from each cell line using the MetaSystems Isis program and the results analyzed using Microsoft Excel. A ratio of combined telomere length to total chromosome length was calculated to normalize the data for all cells. The abnormal and normal cell line measurements were

then compared using a Student's t test via Excel.

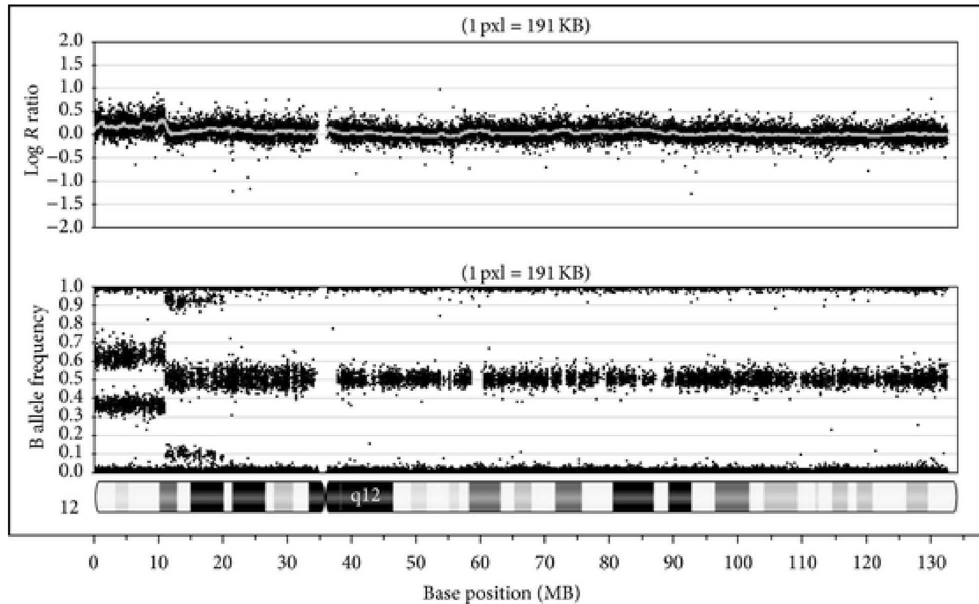
Genome-wide SNP array analysis using Illumina Quad 610 array was performed in the Cytogenomics Laboratory at the Children's Hospital of Philadelphia on DNA extracted from peripheral blood samples. The array contains 28,528 SNP probes on chromosome 12. Log *R* ratios were used to determine the dosage of patient DNA by intensity of signal and parental DNA was analyzed to determine the origin of the duplicated chromosomal segment in the child. B allele frequency was calculated using genotype clusters per SNP as determined from HapMap sample analysis. Methods for SNP array analysis were as previously described.

**Results**

FISH studies confirmed four copies of the TEL (12p13) and subtelomere (12p terminal) probes. The PNA probes used to assess telomere length showed the presence of additional telomere signals on the abnormal 12p. The average telomere ratio for chromosome 12 was 0.034±0.028 for the normal cell line and 0.033±0.044 for the abnormal cell line. A Student's t-test revealed a lack of statistical significance between the telomere lengths of the two cell lines ($p<0.47$).

SNP array analysis indicated that there were 3 copies of 12p11.21 to 12p13.2 with three haplotypes for 12p11.2 to 12p13.2 and mosaicism of 20 percent was computed based on the B allele frequencies.[5,6]. The additional material was identified as maternal in origin through the use of informative SNPs and comparison of parent and child genotypes. At least four copies of 12p13.2 to 12pter were identified with two haplotypes (Figure 1). Our patient's karyotype was interpreted as 46,XX,dup(12)(p11.2p13.2),trp(12) (p13.2pter)[5]/46,XX[15]; twenty-five percent of her cells had three to four copies of 12p (Figure 2); the other seventy-five percent were normal.

a)




b)

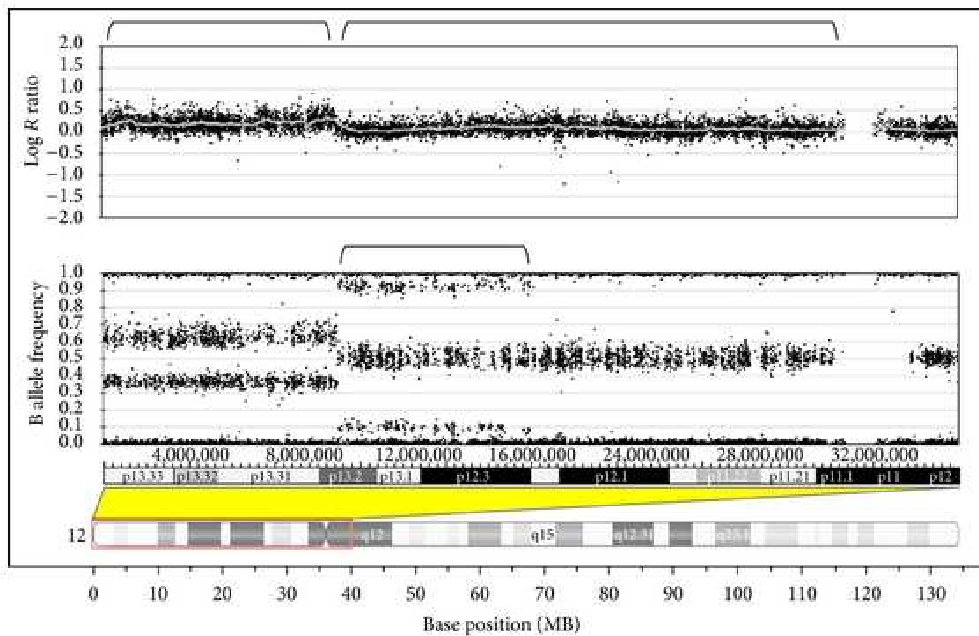



Figure 1: (a) SNP array results for chromosome 12 showing Log *R* ratios in the top panel and B allele frequency in the bottom panel. The long arm of chromosome 12 shows no copy number or genotyping abnormalities. The short arm shows two regions of copy number change, with more copies of the terminal region of 12p and the proximal 12p region. (b) SNP array results for 12p only with the Log *R* ratio in the upper panel and the B allele frequency in the bottom panel. Regions of mosaicism for four copies (terminal) and three copies (proximal) are indicated by brackets. The additional genotypes in the region of mosaicism for three copies are shown by the bracket in the lower panel. This genotyping pattern indicates that the extra copy of 12p in this region contains an additional maternal haplotype. The presence of three haplotypes suggests and origin of the abnormal 12p in meiosis.

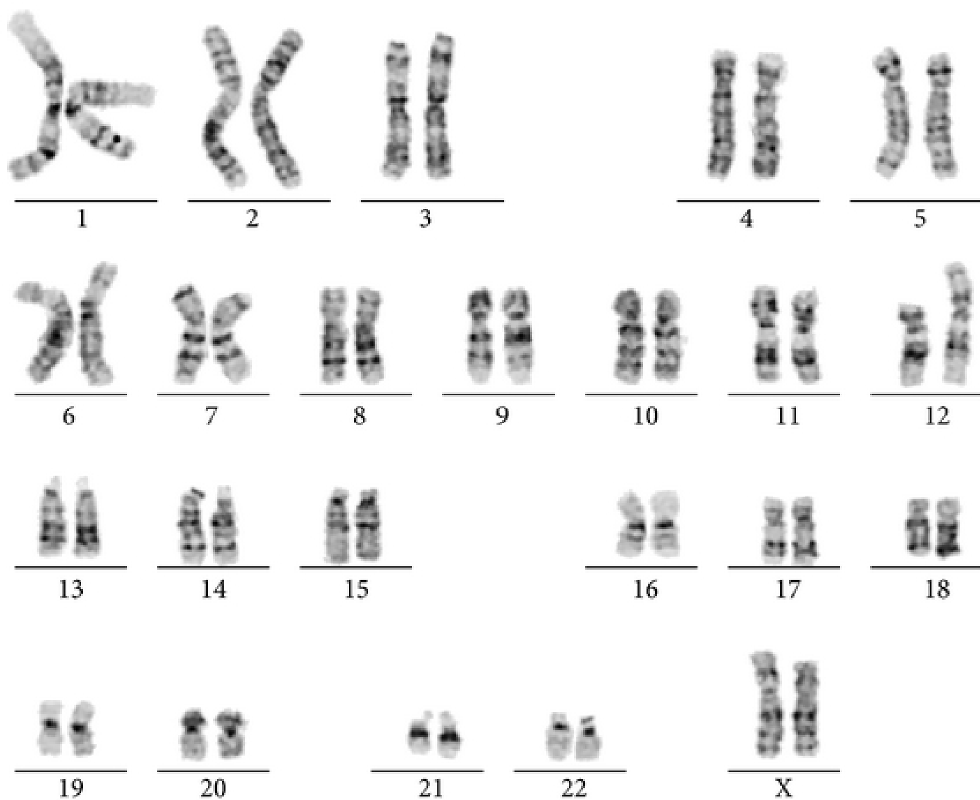



Figure 2: Karyotype of 46,XX,dup(12)(p11.2p13.2),trp(12)(p13pter) seen in 25% of peripheral blood metaphase cells.

**Discussion**

Approximately 26 individuals with varying 12p structural duplications or triplications have appeared in the literature, most shared phenotypic features found in PKS.[4] The similarities in the clinical presentation of our patient to PKS suggested the likelihood of the abnormal cell line completely disappearing from peripheral blood as our patient ages, as tissue-limited mosaicism is a hallmark of PKS presentation. Previously reported cases with 12p duplications (three copies) had the abnormality present in all peripheral blood cells.[4] Two cases with triplications for all of 12p showed tissue limited mosaicism, with the abnormal cell line being present in only skin fibroblasts.[7,8] However, two cases with triplication of 12p regions that did not include 12p13.31 had abnormal cells present in all tissues, including peripheral blood.[9,11] Our patient has four copies of the region proposed to be responsible for the PKS phenotype, 12p13.31, which contains three genes, ING4, CHD4, and MAGP2, responsible for negative growth regulation.[4] Overexpression of ING4 has been shown to result in cell cycle arrest.[12]

Telomere length was evaluated between our patient's two cell lines since it has been reported in mosaic Down syndrome that the trisomic cell line has decreasing telomere length, which may be related to cellular aging.[10] In addition, loss of the abnormal cell line in peripheral blood is a known characteristic of PKS. Shorter telomeres in the abnormal PKS cell line could explain a growth disadvantage that results in this loss; however, we did not see a difference in telomere length between the normal and structurally abnormal cell lines in our patient. This may explain why the abnormal cell line in this patient was not decreasing in the peripheral blood.

Genome-wide SNP array analysis identified three copies of 12p13.2 to 12p11.21 and confirmed the presence of four copies of 12pter to 12p13.2. The additional material was found to be maternal in origin through the use of informative SNPs in the parents (AA v BB). The presence of both a normal cell line and an abnormal cell line with a structural abnormality suggests a mitotic error. However, the SNP results indicated maternal meiotic crossing over, consistent with nondisjunction in meiosis after the crossing over occurred. There were new genotype patterns in the patient from 12p12.2 to 12p13.2, and the remainder of the abnormal 12p arm had triplication of the maternal chromosomal material. This may have occurred during meiosis II, due to the lack of extra genotypes near the centromere. The report of two patients with mosaicism for de novo duplications identified a meiotic error and proposed two trisomy rescue events during mitotic divisions early during embryogenesis.[13]

Izumi and colleagues reported that the critical region for PKS is 12p13.31 based upon a case with an interstitial duplication of 12p and a review of the literature.[3] Our patient had four copies of this region and a PKS phenotype, as expected. However, our patient only has three copies of the 12p11.2 to 12p13.2 region, as opposed to the tetrasomy seen with the isochromosome 12p. While the isochromosome 12p marker is rarely seen in peripheral blood, the abnormal cell line in our patient was present in twenty-five percent of her peripheral blood cells at six and 19 months of age. The isochromosome 12p marker has been reported to be at a higher percentage in interphase than in metaphase cells in peripheral blood from patients with PKS.[14] Our patient, however, has the same percentage of the abnormal cell line in both metaphase and interphase cells. The lack of change in mosaicism in our patient may suggest that the

gene(s) responsible for growth disadvantage in peripheral blood may be located outside the region present in four copies in our patient. Since our patient has at least four copies of the 12p13.31 critical region, this might suggest that the genes that affect cell survival in peripheral blood may be proximal to the 12p13.31 region, which could explain the stability of the abnormal cell line in our patient. This will need to be confirmed by the identification of other PKS patients with mosaicism in peripheral blood.

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# CHAPTER 3: Utilization of FISH to Assess Chromosomal Abnormalities in Pregnancy Loss

## Introduction

Pregnancy loss is relatively common, with 15-20% of recognized pregnancies resulting in spontaneous abortion (SA), most commonly in the first trimester.[1,2] Recurrent miscarriage, defined as the loss of two or more consecutive pregnancies within the first 20-24 weeks of gestation, occurs in 3-5% of couples attempting to bear children.[3,4,5] Determination of the cause of these SAs is useful for counseling patients on future pregnancy planning with regard to recurrence risk and for assisting them in dealing with feelings of guilt or inadequacy that may result from a SA. Fetal chromosomal abnormalities are responsible for 60-70% of all miscarriages.[6,7,8,9] Of these, approximately 95% are numerical abnormalities, with monosomy X, triploidy, and autosomal trisomies chief among them. [1,10]

Between January 2000 and June 2009, a total of 602 products of conception (POC) samples were received in the Cytogenetics Laboratory at Ruby Memorial Hospital. Approximately 26% of these samples failed to grow in culture and an additional 5% were contaminated upon receipt. This is consistent with the literature which shows a 20-25% culture failure rate with POCs.[1,6,10] It is known that the utilization of FISH testing in cases of culture failure and contaminated or otherwise nonviable samples may increase the resulting rate for these POC specimens.[10,11,12] Data on amniotic cell culture failure demonstrates an increased frequency of abnormalities, 10-19% in pregnancies where culture failure occurs versus 1-4% in successful cultures.[13] This study was initiated to obtain results on culture failures to determine if POCs should be processed for FISH in addition to routine culturing upon receipt and then reflexed for FISH testing in the event of a culture failure.

## Materials and Methods

POC samples received from January 2007 through December 2008 were processed according to standard cytogenetics protocol. IRB approval was obtained. Tissue received included placenta, cord, and/or fetus. The blood vessels were stripped

from umbilical cords and cut into pieces to plate on plastic petri dishes. Appropriate selections of fetal tissues were handled in the same manner as the umbilical cord vessels. Villi were teased out of placental samples and separated from any maternal decidua. Once segregated, the villi were minced with a scalpel into pieces small enough to fit through an 18-gauge hypodermic needle. The sample was then placed in 4 mL of 0.05% trypsin EDTA for 10 min at 37°C, spun at 1,500 RPM for 5 min, placed into 4 mL of collagenase for 10 min at 37°C, and then spun again to pellet. At this point, each sample was halved. The portion to be cultured was plated onto glass coverslips contained within plastic petri dishes using AmnioMax karyotyping medium. Ten mL of KCl was added to the remaining pellet and the sample incubated for 10 min at 37°C and then spun. Ten mL of fix (3:1 methanol to glacial acetic acid) was added and the sample refrigerated for future use in FISH studies.

For this study, POCs received in the Ruby Memorial Hospital Cytogenetics Lab from January 2000 through September 2006 and their results were surveyed to determine the most common abnormalities present. FISH was performed on pellets from 17 placental villi samples collected between 2007 and 2008 for the sex chromosomes and also 8, 9, 13-18, and 20-22. Based on the availability of probes, it was decided that the Vysis (Abbott Molecular) AneuVysion kit would be used for the sex chromosomes and 13, 18, and 21, IGH/MYC:CEP 8 for 8 and 14, ASS for 9, PML/RARA for 15 and 17, CBFB for 16, ToTelVysion telomere cocktail #15 for 20, and the TUPLE1 probe for 22.

Samples were processed for FISH as previously described in chapter 2. Four spots per slide were etched and two slides per culture dropped so that each of the 8 probes could be applied separately. Two scorers each read fifty cells per probe for a total of 100 cells.

**Results**

Karyotypes were obtained on 68% of the POCs received between January 2000 and June 2009. Of these, 34.5% were abnormal. Of the remainder, 26% were culture failures and an additional 6% were contaminated or rejected upon receipt. Of the 17 samples available for this project, twelve were for pregnancy losses that grew in culture. FISH confirmed the karyotype results of 8 normal females, 3 normal males, and one

triploid male. Of the 5 remaining samples that could not be karyotyped, one was normal female, one normal male, one was a mosaic tetraploid female, one was a female mosaic for trisomy 9, and the fifth had insufficient cells for FISH analysis.

**Discussion**

Karyotyping results were confirmed in the 12 samples that had been successfully karyotyped and then studied using FISH. This is important given that a majority of POC karyotypes are normal female. There is potential for maternal cell contamination (MCC) in POC specimens, particularly in tissue samples of unidentifiable origin. There can be a tendency to see, when analyzing karyotypes of POCs, a normal female cell line along with an abnormal cell line.[11] In other existing studies, it has been determined that up to 29% of first trimester POCs resulting in a 46,XX karyotype show evidence of Y chromosome presence in interphase cells analyzed by FISH.[14,15] In this study, all 8 of the normal female karyotypes were confirmed by FISH, as were the 3 normal males and the one triploid male.

Of the five samples from tissue culture failure, one was determined to be normal male and one normal female. One mosaic tetraploid female and one trisomy 9 mosaic female were discovered. This may be consistent with more abnormal specimens being less likely to grow in culture. One sample had insufficient cells for FISH analysis. This sample consisted of umbilical cord cells and it has been determined through experience that our FISH processing methods are largely ineffective on cord specimens, as they are difficult to dissociate into single cells. Work is being done currently to improve upon this.

In August 2012, the Cytogenetics Laboratory at Ruby Memorial Hospital, at the request of the Obstetrics and Gynecology Department, implemented a procedural change for the initial set-up of products of conception (POCs) to include processing a portion of each sample for FISH analysis in the event that the tissue fails to grow in culture. Cultures that fail are now automatically reflexed for AneuVysion FISH, which consists of two probe cocktails, one for 18, X, and Y and another for 13 and 21. This is the same probe kit routinely used for prenatal amniotic fluid testing. Additional FISH testing on these samples is done by physician request or to clarify an abnormality seen on the karyotype.

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# CHAPTER 4: Telomere Length and Decreased Lifespan in Newborns with Trisomy 21

## Introduction

Telomeres consist of 500-2000 tandem repeats of the TTAGGG sequence and cap the ends of linear chromosomes. Telomere length is maintained by the telomerase ribonucleoprotein complex, which slows telomere attrition via utilization of the telomerase reverse transcriptase (TERT) and an RNA template, telomerase RNA component (TERC). TERT copies a short region of TERC into telomeric DNA to extend the 3' end of the telomere in cells with high replicative demands, such as lymphocytes. Initial telomere length is implicated in the determination of the lifespan of a cell, as 50-100 telomeric base pairs are lost with each replicative cycle.[1,2] When telomere shortening reaches the critical range of 12.5 units of TTAGGG repeats, on even one chromosome within a cell, that cell no longer proliferates and instead enters senescence or undergoes apoptosis.[3]

Individuals with trisomy 21, or Down syndrome (DS), are known to have a shorter lifespan than chromosomally normal individuals and an increased incidence of age-related health problems such as dementia, osteoporosis, and decreasing immune system function which may be related to an accelerated loss of telomere length with age.[4] Individuals with DS reach 60 years of age on average, compared with 78.5 years in unaffected individuals.[5] It has been reported in mosaic Down syndrome (DS) that the trisomic cell line has decreased telomere length, which may be related to cellular aging and therefore a decreased lifespan in DS individuals.[6] Decreased telomere lengths have been observed in trisomy 21 abortuses, amniocytes, and placentas, as well as in lymphocytes from adult DS patients, but little research exists on telomere lengths in newborn DS patients.[7,8,9,10] The purpose of this study was to examine telomere lengths in peripheral blood samples from newborns with trisomy 21 and compare them with chromosomally normal newborn telomere lengths to ascertain whether the telomere length disparity exists at birth.

**Materials and Methods**

Deidentified, previously cultured peripheral blood samples from three newborns with trisomy 21 and seven newborns with normal karyotypes were obtained for this study. All samples were cultured according to routine cytogenetic procedures as outlined in Chapter 2. Cultured cells were dropped onto slides and assessed for metaphase spreads before being processed for PNA FISH as previously described. The MetaSystems Isis program was used to digitally capture images of each metaphase. The overall length of chromosome 1 and the telomere lengths of 1p and 1q PNA signals were measured manually using the linear measurement tool in Isis for each metaphase cell. A ratio of combined telomere length to total chromosome length was calculated to normalize the results for 20 cells per individual. For interphase cells, telomere lengths were assessed by measuring the intensity of the PNA FITC signals in comparison to the total DAPI fluorescence for 20 cells per individual.

**Results**

The average ratio of telomere to chromosome length in metaphase cells for chromosomally normal individuals was $0.094 \pm 0.027$ and for trisomy 21 individuals was $0.070 \pm 0.017$. A Student's t test demonstrated that telomere length in newborns with trisomy 21 was significantly shorter than in chromosomally normal individuals ($p<0.05$). The average ratio of total telomere to total DNA fluorescence in interphase cells of chromosomally normal individuals was $0.1136 \pm 0.057$ and for individuals with trisomy 21 was $0.0857 \pm 0.071$. The total telomere length for trisomy 21 interphase cells was significantly shorter than in chromosomally normal individuals ($p<0.01$). Table 1 shows data by group and case.

| Table 1: Control Cases Data | | |
|---|---|---|
| **Case Number** | **Average (P + Q)/L** | **ST DEV (P + Q)/L** |
| 178S | 0.071 | 0.022 |
| 179S | 0.092 | 0.021 |
| 231R | 0.100 | 0.021 |
| 348M | 0.104 | 0.026 |
| 812A | 0.071 | 0.027 |
| 824S | 0.100 | 0.028 |
| 844F | 0.105 | 0.019 |
| **Average** | 0.094 | 0.027 |
| **Trisomy 21 Cases Data** | | |
| 246W | 0.078 | 0.023 |
| 313B | 0.065 | 0.011 |
| 842S | 0.067 | 0.014 |
| **Average** | 0.069 | 0.017 |
| (P+Q) is equal to the length of the long arm plus the short arm telomeres for chromosome 1. L is total length of the chromosome. | | |

**Discussion**

Previous studies have shown that there are decreased telomere lengths in fetuses with DS prior to birth, with noted uncertainty as to whether these pregnancies would have continued to term.[8] Loss of pregnancies with DS could correlate with accelerated telomere attrition in the fetus. In this study, the telomere lengths in newborns with DS were significantly shorter than in newborns with apparently normal chromosomes, regardless of whether the telomere lengths were measured in interphase or metaphase cells. Our results were, however, greatly limited by sample size, as there were only three newborns with DS and seven normal newborn fixed pellet samples available. However, our results suggest that the shortened telomeres seen in older individuals with DS were present at birth.

It would be informative to compare telomere lengths between patients with DS and control individuals in a much larger study over a prolonged period of time to

determine whether the individuals with trisomy 21 suffer from an increased telomere attrition rate over the course of the lifespan of the individuals. Research has shown that there is an accelerated rate of telomere loss in adult DS individuals.[4] Accelerated telomere attrition could explain the shorter lifespan and increased incidence of premature age-related illnesses and conditions observed in DS individuals, as accelerated telomere loss has been implicated in the premature immunosenescence and dementia commonly seen in DS patients.[11]

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## Conclusions

FISH has several advantages over conventional cytogenetics procedures in that it can be used on nonliving cells, on interphase cells, and is able to identify and assist in the characterization of abnormalities that cannot be visualized using non-molecular cytogenetics techniques such as karyotyping. There are a variety of instances when karyotyping may fail to provide diagnostic or prognostic information to the clinician, such as occurs with culture failure with products of conception samples, microdeletions in newborns with congenital abnormalities, gene deletions, amplifications, rearrangements, and cryptic translocations in oncology patients, as well as rapid preliminary assessment of aneuploidy in prenatal samples.

In Chapter 1, slides from 33 paraffin-embedded tissues on patients with SCLC were assessed using 5p and 5q subtelomere probes to identify 5q chromosomal deletions with relation to histological findings. Previous studies had associated del(5q) with advanced disease stage and poor survival rates,[1,2,3] which our study did not confirm. There were no differences between the del(5q) cohort and those without the deletion with regard to sex, average post-diagnosis survival time, or staging level at diagnosis. The del(5q) group was older on average, had a higher number of pack-years smoked, had more varied symptoms upon diagnosis, and were more likely to be diagnosed incidentally. Of statistical significance was the correlation between del(5q) and spindle cell morphology ($p<0.025$). There were no significant differences seen between the groups with regard to mitotic cell count, degrees of necrosis or fibrosis, mucin production, or neuroendocrine morphology. The significance of variation in spindle cell morphology with regard to del(5q) is unclear at this time. The use of a subtelomere probe to assess del(5q) leaves room for uncertainty in that it can identify terminal deletions, but could fail to detect interstitial deletions. However, this project would not have been possible without the utilization of FISH techniques on paraffin embedded slides since tissue was not available for conventional cytogenetics.

In Chapter 2, the 12p region of a patient with mosaicism for additional 12p material was studied using centromere 12, subtelomere 12p, and the TEL (12p13) probes in conjunction with SNP array analysis. The patient's karyotype was defined as 46,XX,dup(12)(p11.2p13.2),trp(12)(p13.2pter)[5]/46,XX[15] using this combination of

techniques. Our patient had four copies of the region proposed to be responsible for the Pallister-Killian (PKS) phenotype 12p13.31, containing three genes responsible for negative growth regulation, ING4, CHD4, and MAGP2.[4] Izumi and colleagues reported that the critical region for PKS is 12p13.31 based upon a case with an interstitial duplication of 12p and a review of the literature.[5] Our patient had four copies of this region and a PKS phenotype, as expected. However, our patient only had three copies of the 12p11.2 to 12p13.2 region, as opposed to the tetrasomy (four copies) seen with the isochromosome 12p. Telomere lengths were compared between the normal and abnormal cell lines, given that a hallmark of PKS is the disappearance of the abnormal cell line in peripheral blood over time. Shorter telomeres may account for this, however we did not see a difference between telomere lengths in the normal and structurally abnormal cell lines in our patient between six and 19 months of age. While the isochromosome 12p marker is rarely seen in peripheral blood, the abnormal cell line in our patient was present in twenty-five percent of her peripheral blood cells in both metaphase and interphase cells at six and 19 months of age. The isochromosome 12p marker has been reported to be at a higher percentage in interphase than in metaphase cells in peripheral blood from patients with PKS.[6] The lack of change in mosaicism in our patient may suggest that the gene(s) responsible for growth disadvantage in peripheral blood may be located outside the region present in four copies in our patient. Since our patient has at least four copies of the 12p13.31 critical region, this might suggest that the genes that affect cell survival in peripheral blood may be proximal to the 12p13.31 region, which could explain the stability of the abnormal cell line in our patient. This will need to be confirmed by the identification of other PKS patients with mosaicism in peripheral blood. In this instance, a combination of traditional cytogenetics and molecular techniques- FISH and SNP array, were necessary in order to evaluate the patient fully, beyond the abilities of traditional karyotyping. FISH and microarray data defined the number of copies of 12p regions and narrowed the region that may be responsible for cell survival in the tissue-limited mosaic condition of Pallister-Killian syndrome.

In Chapter 3, 17 products of conception (POC) samples were evaluated for numerous chromosomes, including X and Y, 8, 9, 13, 14, 15, 16, 17, 18, 20, 21 and 22, as these are common trisomies associated with pregnancy losses.[7] FISH confirmed the

karyotype results of 8 normal females, 3 normal males, and one triploid male. Five of these samples failed to grow in culture and FISH probes determined that one was a normal female, one a normal male, one a mosaic tetraploid female, one was mosaic for trisomy 9, and the fifth had insufficient cells for FISH analysis. The mosaic trisomy 9 and mosaic tetraploid that failed to grow may be consistent with more abnormal cell lines being less likely to grow in culture or resulting in non-viability of fetus, resulting in pregnancy loss. Of note, there is potential for maternal cell contamination in POC specimens and therefore a tendency to see, when analyzing karyotypes of POCs, a normal female cell line along with an abnormal cell line.[8] In existing studies, it has been determined that up to 29% of first trimester POCs resulting in a 46,XX karyotype show evidence of Y chromosome presence in interphase cells analyzed by FISH.[9,10] In these cases, FISH can assist with assessment of potentially masked abnormalities when a 46,XX karyotype is obtained, which may represent maternal cells. In addition, in incidences of culture failure or contamination, when it is not possible to successfully obtain a karyotype, FISH may be utilized in the determination of fetal abnormalities where answers would not otherwise be obtained.

In Chapter 4, telomere lengths in metaphase and interphase cells were compared between newborns with trisomy 21, or Down syndrome (DS) and normal chromosomes using PNA FISH probes. Our study demonstrated that patients with DS had significantly ($p<0.05$) shorter telomeres in metaphase cells and interphase cells ($p<0.01$), than newborns with normal karyotypes. Other studies have shown such a difference in fetuses with DS and in adults with DS.[11,12] Shorter telomeres in patients with DS, along with increased telomere attrition rates, have been implicated in the shorter lifespan and premature presence of age-related illnesses commonly seen in DS individuals, such as dementia, osteoporosis, and immune system failure.[13] In this case, PNA probes allowed us to see and measure what we otherwise could not with conventional cytogenetics.

Overall, FISH enhances the evaluative and diagnostic possibilities within the scope of cytogenetics testing. It can be used to obtain results when conventional cytogenetics fails, as with nonliving cells, in cases of culture failure, when the abnormality in question is unable to be seen in a karyotype, or a rapid turn around time is desired. FISH can be used to look at specific loci on a sequential level without being as

cumbersome or expensive as array or sequencing techniques. As our studies show, FISH can be useful to identify genes or regions that may be responsible for specific patient characteristics or cellular behavior. Disadvantages for interphase analysis include the inability to visualize FISH results in the context of a metaphase and limitation of the information relative to the specific probe. However, these downsides fail to detract from the overall usefulness of the technique.

There were a few issues within each project that arose with regard to availability of samples and FISH probes for testing. For example, in Chapter 1 subtelomere probes were used to determine 5q deletions. Terminal probes would fail to detect interstitial 5q deletions that may have been present. This project was also limited by sample size. A more informative approach would be to reassess non-deleted cases for interstitial deletions using locus specific probes for 5q genes of interest. Results in Chapter 2 will require confirmation via the identification of additional patients with PKS who exhibit mosaicism for a structural abnormality in peripheral blood. Results from Chapter 3 were greatly limited by availability of cell pellets at the time of the study. It would be more informative to continue this study over the long term with a much larger sample size and track results on culture failures and 46,XX results for several years to determine the true effectiveness of the technique. To expand upon Chapter 4 results, a more informative study would have a larger sample size and would involve tracking telomere length changes over time for each individual. This would assist in confirming whether the individuals with trisomy 21 experience increased telomere attrition rates over the course of a lifespan.

In summation, FISH techniques can provide additional information that cannot be determined by karyotypes, such as the visualization of a single gene locus and otherwise cryptic deletions, amplifications, translocations, or rearrangements. FISH can be used when a karyotype is unobtainable, such as with non-dividing or interphase cells or paraffin-embedded tissues. These techniques can be used to identify chromosomal abnormalities associated with abnormal phenotypes, confirm/clarify a diagnosis, assist with the determination of prognostic status, or help to delineate genes involved in abnormal phenotypes.

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# Appendix

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# Del(5q) Is Associated With Clinical and Histological Parameters in Small Cell Neuroendocrine Lung Carcinoma

Paul H. Hartel, MD, Amy L. Shackelford, CLSp(CG), James V. Hartel, MD, and Sharon L. Wenger, PhD

To elucidate the relationship between del(5q) and the clinical and histological features of small cell neuroendocrine lung carcinoma, 33 tissue samples from patients with this tumor were evaluated. By using fluorescence in situ hybridization, del(5q) was identified in almost 50% of cases (15/33, 45%). Clinically, patients with tumors showing del(5q) were older (mean age = 71 years) with a correspondingly greater pack-year smoking history (mean = 61) than patients with tumors (mean age = 59 years, mean pack-years = 44) without del(5q). Histologically, tumors with del(5q) had a greater frequency of spindle cell morphology (11/14 [79%] vs 6/16 [38%], $P < .025$) than those without del(5q). This is the first study to find an association between del(5q) and tumor histology in small cell neuroendocrine lung carcinoma.

**Keywords:** chromosome; 5q; small cell; neuroendocrine; lung cancer; spindle cell

Lung cancer is a leading cause of cancer deaths in the United States, with the carcinogens in tobacco smoke playing a major etiological role.[1] Genetic changes responsible for carcinogenesis include activation of proto-oncogenes and inactivation of tumor suppressor genes.[2] Tumor suppressor gene inactivation is contributed to, in part, by loss of chromosomal DNA.[3] Although cytogenetic findings in small cell neuroendocrine lung carcinoma are complex, 5q deletion (del(5q)) is the most frequently identified among them.[4,5] Few studies have investigated the relationship between cytogenetic alterations and clinical factors in small cell neuroendocrine lung carcinoma.[5-7] We reviewed 33 cases of small cell neuroendocrine lung carcinoma to elucidate the relationship between del(5q) and the clinical and histological features of this very aggressive tumor.

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## Materials and Methods

Institutional review board approval was obtained for this retrospective study. Thirty-six cases diagnosed as small cell carcinoma of the lung from 1998 to 2005 were retrieved from the archives of the West Virginia University Department of Pathology. Clinical and follow-up data were obtained from patient records. Hematoxylin and eosin (H&E) stained sections were available for each case (range = 1 to 14; mean = 3). Histological diagnosis was confirmed by 2 pathologists (P.H.H., J.V.H) using World Health Organization criteria. Tumor histomorphological features were reviewed, including tumor cell morphology (spindle versus ovoid cells), mitotic count, degree of necrosis, degree of fibrosis, mucin production, and neuroendocrine morphology (rosette formation and peripheral palisading). Tumors were considered to have spindle cell morphology if 50% or more of tumor cells showed this feature. Mitoses were counted as the mean per high power field (minimum of 30 fields counted). Tumor necrosis was graded as absent, present in less than or equal to 50% of the tumor biopsy, or present in greater



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than 50% of the tumor biopsy. Fibrosis, mucin production, and neuroendocrine morphology were assessed as either present or absent. Mucin production was assessed using periodic acid Schiff histochemistry and included tumor intracellular mucin and extracellular matrix mucin within tumors. All but 3 of the initial 36 cases were successfully analyzed by fluorescence in situ hybridization (FISH) for a total of 33 cases included in the study group.

## Cytogenetics

In all, 3-mm thick tissue was placed on glass microscopic slides. The abnormal areas on one H&E slide per case were marked by 2 pathologists (P.H.H, J.V.H). Unstained slides were etched to reflect the H&E marking. To prepare for FISH, 1 slide per case was deparaffinized using SkipDewax (Insitus Biotechnologies, Albuquerque, New Mexico) at 80°C for 20 minutes followed by two 3-minute distilled water washes. Slides were then placed in a pepsin solution (25 mg pepsin, 49.5 mL distilled $H_2O$, 0.5 mL 1N HCl, pH = 2.0) for 1 hour at 37°C followed by two 5-minute washes in 2 × sodium chloride/sodium citrate (SSC, pH 7.0). The slides were dried on a slide warmer at 50°C for 5 minutes.

Subtelomere probes for 5p and 5q (Vysis TotelVysion cocktail #5; Abbott Molecular Inc, Downers Grove, Illinois) were diluted (2:50 µL) in tDenHyb (Insitus Biotechnologies, Albuquerque, New Mexico). This probe mixture was applied to the previously etched area of the tissue in an amount relative to the size of the sample and sealed under a cover glass using rubber cement, typically 5 µL of probe under an 18-mm round glass. Slides were placed in a ThermoBrite slide warmer (Abbott Molecular Inc, Des Plaines, Illinois) at 90°C for a 3-minute denaturing time. They were then placed in sealed, moist slide boxes in a 37°C water bath to hybridize for 16 to 20 hours.

After hybridization, rubber cement and cover glasses were removed, and slides were washed in 2 × SSC, 0.3% NP-40 (pH 7.0) at 72°C for 2.5 minutes and then allowed to dry in the dark for 10 minutes. In all, 20 mL of 4',6-diamidino-2-phenylindole (DAPI) counterstain was then applied to each slide and sealed with a cover glass of 20 × 50 mm.

A Leica epi-fluorescent microscope equipped with a DAPI single band-pass and a red/green dual band-pass filters was used for signal enumeration of red and green signals. Up to 100 nonoverlapping cells were scored by 2 individuals (A.L.S., S.L.W) with the 5q telomere indicated by the red signal and the 5p telomere region by green. Any red to green ratio under 0.80 was considered a 5q deletion. Statistical analyses were performed using Student *t* test and chi-squared goodness of fit test.

**Table 1.** Clinical Findings in Patients With Small Cell Lung Cancer

| Age, mean, y | 75 |
|---|---|
| Sex | |
| Male, % | 52 (17/33) |
| Female, % | 48 (16/33) |
| Symptoms | |
| Dyspnea, % | 36 (11/30) |
| Cough, % | 17 (5/30) |
| Incidental, % | 17 (5/30) |
| Other, % | 30 (9/30) |
| Pack-year smoking history | 49 |
| Tumor location | |
| RUL, % | 46 (15/33) |
| RML, % | 9 (3/33) |
| RLL, % | 9 (3/33) |
| LUL, % | 21 (7/33) |
| LLL, % | 6 (2/33) |
| Right lung, lobe not specified, % | 6 (2/33) |
| RML and RLL, % | 3 (1/33) |
| Procedure | |
| Transbronchial biopsy, % | 82 (27/33) |
| Open biopsy, % | 12 (4/33) |
| Lobectomy, % | 6 (2/33) |
| Clinical disease stage at diagnosis | |
| 1, % | 10 (3/30) |
| 2, % | 7 (2/30) |
| 3, % | 16 (5/30) |
| 4, % | 67 (20/30) |
| Treatment | |
| Surgery only, % | 7 (2/30) |
| Surgery and chemo and/or radiation, % | 13 (4/30) |
| Chemo and/or radiation only, % | 80 (24/30) |
| Outcome | |
| Alive, disease status unknown, % | 13 (4/31) |
| Alive, no evidence of disease, % | 3 (1/31) |
| Dead of disease, % | 84 (26/31) |
| Survival, mean, mo | 21 |

NOTES: RUL = right upper lobe; RML = right middle lobe; RLL = right lower lobe; LUL = left upper lobe; LLL = left lower lobe.

## Results

Pertinent clinical findings of the overall study group are presented in Table 1. Table 2 shows clinical findings by del(5q) status. No cases had clinical or radiological evidence of a nonpulmonary primary tumor site.

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**Table 2.** Clinical Findings of Patients With or Without Del(5q)

| | With Del(5q) | Without Del(5q) |
|---|---|---|
| Age, mean, y[a] | 71 | 59 |
| Sex | | |
| Male, % | 53 (8/15) | 50 (9/18) |
| Female, % | 47 (7/15) | 50 (9/18) |
| Symptoms | | |
| Dyspnea, %[b] | 27 (4/15) | 41 (7/17) |
| Cough, %[c] | 27 (4/15) | 6 (1/17) |
| Incidental, % | 33 (5/15) | 0 (0/17) |
| Other, % | 13 (2/15) | 53 (9/17) |
| Pack-year smoking history, mean[d] | 61 | 44 |
| Tumor location | | |
| Right lung, % | 73 (11/15) | 72 (13/18) |
| Left lung, % | 27 (4/15) | 28 (5/18) |
| Clinical disease stage at diagnosis | | |
| 1, 2, or 3, % | 38 (5/13) | 29 (5/17) |
| 4, % | 62 (8/13) | 71 (12/17) |
| Survival, mean, mo | 20 | 21 |

[a]Age, $P < .003$.
[b]Dyspnea, $P < .5$.
[c]Cough, $P < .1$.
[d]Pack-year history, $P < .02$.

Terminal deletions of 5q were identified in 45% of cases (15/33; Figure 1, Table 2). Patients with tumors containing del(5q) included 8 men and 7 women ranging in age from 56 to 83 years (mean = 71). Although the most common presenting symptoms were dyspnea and cough, the most frequent clinical presentation in this group was as an incidental finding (33%, 5/15). Smoking history ranged from 30 to 88 pack-years (mean = 61). Tumors were located more often in the right lung (11/15, 73%) than left (4/15, 27%). Clinical stages of disease were stage 1, 2, or 3 (5/13, 38%) and stage 4 (8/13, 62%). Survival ranged from 1 to 67 months (mean = 20).

Patients with tumors without del(5q) included 9 men and 9 women ranging from 37 to 84 years of age (mean = 59). The most common presenting symptom was dyspnea (7/17, 41%). Smoking history ranged from 10 to 65 pack-years (mean = 44). Similar to patients with del(5q), tumors were located more often in the right lung (13/18, 72%) than left (5/18, 28%). Clinical stages of disease were stage 1, 2, or 3 (5/17, 29%) and stage 4 (12/17, 71%), and survival ranged from 2 to 86 months (mean = 21).

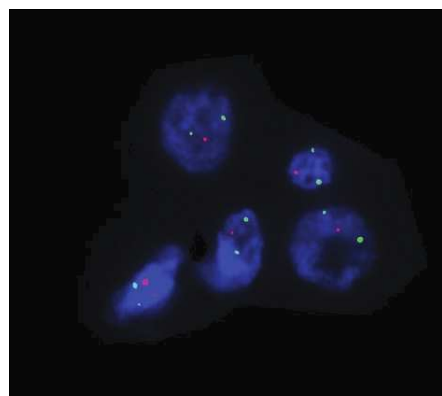

**Figure 1.** Fluorescence in situ hybridization showing small cell neuroendocrine lung carcinoma tumor cells with 2 green signals and only 1 red signal (del(5q)). One tumor cell (lower left) shows 2 green and 2 red signals (absent del(5q)).

Pertinent histological findings and del(5q) status are presented in Table 3. Spindle cell morphology was more commonly seen in tumors with del(5q) (11/14, 79%; Figure 2) than in tumors without del(5q) (6/16, 38%), which was significantly different ($P < .025$). Mucin production was identified more often in tumors (6/15, 40%; Figure 3) with del(5q) than in tumors (5/17, 29%) without del(5q); however, this was not statistically different ($P < .9$). Histological findings of mitotic count, necrosis, fibrosis, and neuroendocrine morphology did not differ between tumors with or without del(5q).

## Discussion

Specific cytogenetic abnormalities have been associated with small cell neuroendocrine lung carcinoma, and del(5q) is among the most commonly reported.[4,5] However, very few studies have evaluated the relationship between del(5q) and clinical parameters.[5-7] There are no studies to our knowledge that have assessed associations between del(5q) and histomorphology in small cell neuroendocrine lung carcinoma. We evaluated 33 cases of small cell neuroendocrine lung carcinoma to elucidate the relationship between del(5q) and the clinical and histological features of this tumor.

Since the initial report of a nonrandom chromosomal abnormality, del(3p), in tumor cells from

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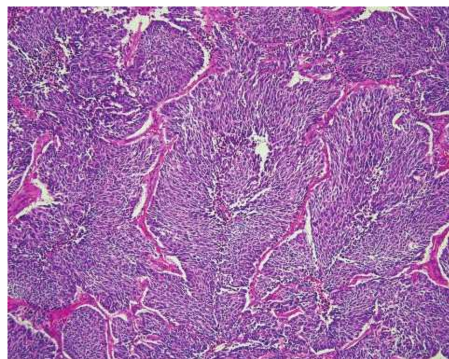

**Figure 2.** Small cell neuroendocrine lung carcinoma with spindle cell morphology. hematoxylin and eosin, medium power.

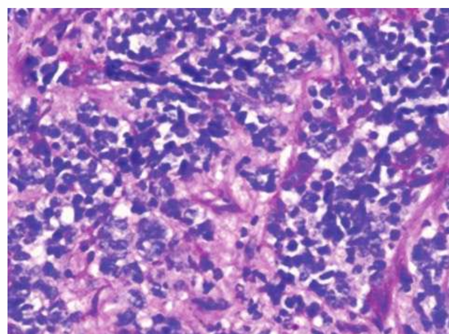

**Figure 3.** Small cell neuroendocrine lung carcinoma with mucin secretion. Periodic acid Schiff, high power.

patients with small cell neuroendocrine lung carcinoma,[8] losses on chromosomes 3p, 5q, 13q, and 17p have been recurrent findings in small cell and non-small cell lung neoplasms.[9-11] Along with del(3p), del(5q) is the most common cytogenetic abnormality reported in small cell neuroendocrine lung carcinoma.[4,12,13] Deletions involving 5q in lung cancer include the APC/MCC tumor suppressor gene cluster, the 5q33–q35 region, the 5q11–q13 region containing the mismatch repair gene hMSH3[14] and deletions involving 5q31.[15] Six cases that did not have a terminal 5q deletion were tested using a FISH probe for EGR1 locus at 5q31, which was not

**Table 3.** Tumor Histology in Small Cell Lung Cancer and Del(5q)

| | With Del(5q) | Without Del(5q) |
|---|---|---|
| Spindle cell morphology, %[a] | 79 (11/14) | 38 (6/16) |
| Mucin production, %[b] | 40 (6/15) | 29 (5/17) |

[a]Spindle cell morphology, $P < .025$.
[b]Mucin, $P < .9$.

deleted in any of these cases. Although a few studies have reported del(5q) to be associated with advanced disease stage[5,6] and poor survival,[7] other studies have not found associations between del(5q) and clinical variables including sex, metastasis, or survival,[9] consistent with our findings. No studies have investigated associations with del(5q) and tumor histomorphology.

In our study of 33 cases of small cell neuroendocrine lung carcinoma, we found clinical and histological associations with del(5q). Patients with tumors having del(5q) were older than patients without del(5q) and had greater pack-year smoking histories likely explained by their older age. That the patients with tumors having del(5q) were older suggests that del(5q) may be a result of longer exposure to mutagenic influences, that is smoking, or that del(5q) occurs as a later "hit" in the tumorigenic pathway of small cell neuroendocrine lung carcinoma in some patients. Conversely, the absence of del(5q) being associated with a younger patient age may suggest an inherently more aggressive tumor that manifests at earlier age. Chromosomal losses have been shown to have meaningful clinical correlates, whether prognostically favorable or unfavorable, in oligodendroglioma (loss of 1p and 19q),[16] myelodysplastic syndromes (loss of 5q, 7q, and 20q),[17] acute myeloid leukemia (loss of 5q and 9q),[18] and chronic lymphocytic leukemia (loss of 13q).[19] Furthermore, all our cases without del(5q) were symptomatic at the time of diagnosis. This is in contrast to cases with del(5q) where one-third were asymptomatic and diagnosed as an incidental finding. Although the older patients in this group may have been more likely to have their tumors discovered incidentally as they sought medical attention for other conditions, it may be that del(5q) indicates a biologically less aggressive tumor characterized by later onset and less symptomatology.

We also found histological associations with del(5q) in small cell neuroendocrine lung carcinoma. Tumors with del(5q) had more than a 2-fold greater

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frequency of spindle cell morphology than tumors without del(5q), which was statistically significant ($P < .025$). Tumors without del(5q) more often had round to ovoid tumor cells. Tumors with del(5q) also showed more frequent mucin production. Interestingly, when symptomatic, patients in this group more often reported cough as the presenting symptom than patients with tumors without del(5q). The greater frequency of cough in patients with tumors showing del(5q) may be related to the more frequent mucin production. When interpreting the results of our study, it is important to note that the vast majority of our tissue was obtained from transbronchial biopsies. The histological and cytogenetic results are limited to the extent that the small biopsies may not be wholly representative of the larger tumor. Additionally, the results are limited by the study sample size.

The present study evaluated 33 cases of small cell neuroendocrine lung carcinoma to elucidate the relationship between del(5q) and the clinical and histological features of small cell neuroendocrine lung carcinoma. The chromosomal abnormality del(5q) was associated with older patients with greater pack-year smoking history, spindle cell tumor morphology, and mucin secretion. Future studies are warranted to confirm the cytogenetic associations with clinical and histological variables in small cell neuroendocrine lung carcinoma using larger sample sizes, larger tumor samples (ie, wedge resections), and more extensive cytogenetic analyses.

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*Case Report*

# Persistent Mosaicism for 12p Duplication/Triplication Chromosome Structural Abnormality in Peripheral Blood

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We present a rare case of mosaicism for a structural abnormality of chromosome 12 in a patient with phenotypic features of Pallister-Killian syndrome. A six-month-old child with dysmorphic features, exotropia, hypotonia, and developmental delay was mosaic for both a normal karyotype and a cell line with 12p duplication/triplication in 25 percent of metaphase cells. Utilization of fluorescence in situ hybridization (FISH) identified three copies of probes from the end of the short arm of chromosome 12 (TEL(12p13) locus and the subtelomere (12p terminal)) on the structurally abnormal chromosome 12. Genome-wide SNP array analysis revealed that the regions of duplication and triplication were of maternal origin. The abnormal cell line in our patient was present at 25 percent at six months and 19 months of age in both metaphase and interphase cells from peripheral blood, where typically the isochromosome 12p is absent in the newborn. This may suggest that the gene(s) resulting in a growth disadvantage of abnormal cells in peripheral blood of patients with tetrasomy 12p may not have the same influence when present in only three copies.

## 1. Introduction

Patients with trisomy 12p typically present with severe mental retardation, seizures, low-set ears, and characteristic facial dysmorphology including flatness of the face, small nose with broad bridge, anteverted nares, inner epicanthal folds, long philtrum, everted lower lip, and high forehead. The extra copy is due to an intrachromosomal duplication or an extra copy on a derivative chromosome. Patients with tetrasomy 12p, or Pallister-Killian syndrome (PKS), additionally present with sparse temporal hair, eyebrows, and eyelashes, prominent forehead, a cupid-bow shaped mouth, and large ears. A hallmark of PKS is tissue-limited mosaicism, with few, if any, abnormal cells found in peripheral blood lymphocyte metaphases in the newborn. Another characteristic of tetrasomy 12p is loss of the abnormal cell line in peripheral blood and skin fibroblasts as the patient ages or over time in serial-passaged cultured fibroblasts [1, 2].

We report on a patient with mosaicism for duplication and triplication of 12p. Only about 24 cases with mosaicism for a structural abnormality of an autosome have been reported in the literature [3]. Approximately 26 individuals with nonmosaic structural 12p duplications have been reported in the literature with a minimum critical region of 12p13.31 [4]. We report on a patient with mosaicism in peripheral blood for a derivative chromosome involving duplication and triplication of 12p.

## 2. Clinical Report

Our patient was delivered at 38 weeks gestation to a 19-year-old mother. The pregnancy was complicated by gestational diabetes and polyhydramnios. At birth, the infant presented with an anal fistula, hypertension, mild hypotonia, ventricular septal defect (VSD), and intraventricular hemorrhage

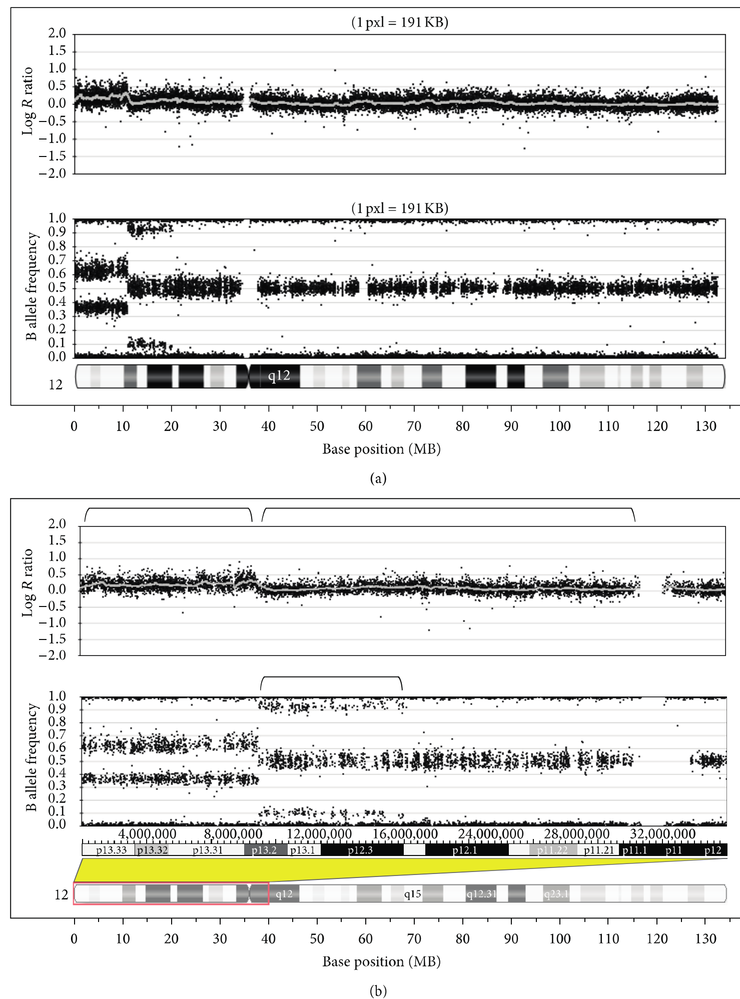

Figure 1: (a) SNP array results for chromosome 12 showing Log $R$ ratios in the top panel and B allele frequency in the bottom panel. The long arm of chromosome 12 shows no copy number of genotyping abnormalities. The short arm shows two regions of copy number change, with more copies of the terminal region of 12p and the proximal 12p region. (b) SNP array results for 12p only with the Log $R$ ratio in the upper panel and the B allele frequency in the bottom panel. Regions of mosaicism for four copies (terminal) and three copies (proximal) are indicated by brackets. The additional genotypes in the region of mosaicism for three copies are shown by the bracket in the lower panel. This genotyping pattern indicates that the extra copy of 12p in this region contains an additional maternal haplotype. The presence of three haplotypes suggests an origin of the abnormal 12p in meiosis.

and was hospitalized for 3 and a half weeks due to breathing difficulties. At 5 months of age, the patient was able to roll over and sit with support and had exotropia. She was seen by genetics at 6 months of age and was found to be dysmorphic and hypotonic with significant developmental delays. At ten months, she was unable to crawl or sit without support. Her height and weight were in the 50th percentile, with head circumference at the 90th percentile. At 19 months,

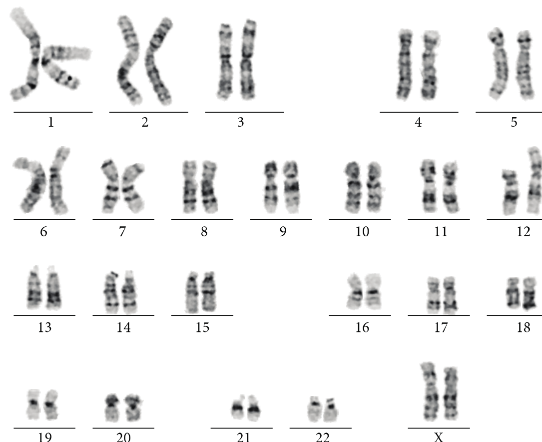


Figure 2: Karyotype of 46,XX,dup(12)(p11.2p13.2),trp(12)(p13.2 pter) seen in 25% of peripheral blood metaphase cells.

the patient had a broad, high forehead, bitemporal balding, small posteriorly rotated ears, global developmental delays, and mild hypotonia. She could sit but not pull to a stand. She was asymptomatic for VSD, had eye surgery to remove chalazia, and was receiving physical, speech, and vision therapies as well as seeing a developmental specialist.

## 3. Materials and Methods

Peripheral blood, obtained from our patient and her parents, was processed using routine cytogenetic procedures to obtain a karyotype and a DNA extraction for microarray analysis. FISH was performed on the patient's peripheral blood metaphases using the centromere 12, TEL (12p13), and subtelomere 12p probes from Vysis (Abbott Molecular, Inc., Downers Grove, IL). All probes were diluted (2 $\mu$L : 50 $\mu$L) in *c*DenHyb (InSitus Biotechnologies, Albuquerque, NM) and hybridized to the target locations on chromosome 12 following the manufacturer's protocol. One hundred cells were scored on each probe to determine the percentages of normal and abnormal cells.

Genome-wide SNP array analysis using Illumina Quad 610 array was performed in the Cytogenomics Laboratory at the Children's Hospital of Philadelphia on the extracted DNA. The array contains 28,528 SNP probes on chromosome 12. Log *R* ratios were used to determine the dosage of patient DNA by intensity of signal, and parental DNA was analyzed to determine the origin of the duplicated chromosomal segment in the child. B allele frequency was calculated using genotype clusters per SNP as determined from HapMap sample analysis. Methods for SNP array analysis were as previously described, and mosaicism of 20% was computed based on the B allele frequencies [5, 6].

## 4. Results

FISH studies confirmed four copies of the TEL (12p13) and subtelomere (12p terminal) probes. SNP array analysis indicated that there were 3 copies of 12p11.21 to 12p13.2 with three haplotypes for 12p11.2 to 12p13.2. The additional material was identified as maternal in origin through the use of informative SNPs and comparison of parent and child genotypes. At least four copies of 12p13.2 to 12pter were identified with two haplotypes (Figure 1). Our patient's karyotype was interpreted as 46,XX,dup(12)(p11.2p13.2),trp(12)(p13.2pter)[5]/46,XX[15]; twenty-five percent of her cells had three to four copies of 12p (Figure 2); the other seventy-five percent were normal.

## 5. Discussion

Approximately 26 individuals with varying 12p structural duplications or triplications have appeared in the literature [4], most shared phenotypic features found in PKS. These similarities in the clinical presentation of our patient to PKS suggested the likelihood of the abnormal cell line completely disappearing from peripheral blood as our patient ages, as tissue-limited mosaicism is a hallmark of PKS presentation. Previously reported cases with 12p duplications (three copies) [4] were present in all peripheral blood cells. Two cases with triplications for all of 12p showed tissue limited mosaicism, with the abnormal cell line being present in only skin fibroblasts [7, 8]. However, two cases with triplication of 12p regions that did not include 12p13.31 were present in all tissues, including peripheral blood [9, 10]. Our patient has four copies of the region proposed to be responsible for the PKS phenotype, 12p13.31, which contains three genes, ING4, CHD4, and MAGP2, responsible for negative growth

regulation [4]. Overexpression of ING4 has been shown to result in cell cycle arrest [11].

Genome-wide SNP array analysis identified three copies of 12p13.2 to 12p11.21 and confirmed the presence of four copies of 12pter to 12p13.2. The additional material was found to be maternal in origin through the use of informative SNPs in the parents (AA v BB). The presence of both a normal cell line and an abnormal cell line with a structural abnormality suggests a mitotic error. However, the SNP results indicated maternal meiotic crossing over, consistent with nondisjunction in meiosis after the crossing over occurred. There are new genotype patterns in the patient from 12p12.2 to 12p13.2, and the remainder of the abnormal 12p arm had triplication of the maternal chromosomal material. This may have occurred during meiosis II, due to the lack of extra genotypes near the centromere. The report of two patients with mosaicism for de novo duplications identified a meiotic error and proposed two trisomy rescue events during mitotic divisions early during embryogenesis [12].

Izumi and colleagues [4] reported that the critical region for PKS is 12p13.31 based upon a case with an interstitial duplication of 12p and a review of the literature. Our patient had four copies of this region and a PKS phenotype, as expected. However, our patient only has three copies of the 12p11.2 to 12p13.2 region, as opposed to the tetrasomy seen with the isochromosome 12p. While the isochromosome 12p marker is rarely seen in peripheral blood, the abnormal cell line in our patient is present in twenty-five percent of her peripheral blood cells at six and 19 months of age. The isochromosome 12p marker has been reported to be at a higher percentage in interphase than in metaphase cells from patients with PKS [13]. Our patient, however, has the same percentage of the abnormal cell line in both metaphase and interphase cells. The lack of change in mosaicism in our patient may suggest that the gene(s) responsible for growth disadvantage in peripheral blood may be located outside the region present in four copies in our patient. Since our patient has at least four copies of the 12p13.31 critical region, this might suggest that the genes that affect cell survival in peripheral blood may be proximal to the 12p13.31 region, which would explain the stability of the abnormal cell line in our patient. This will need to be confirmed by the identification of other PKS patients with mosaicism in peripheral blood.

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Letter to the Editor

**Decreased telomere length in metaphase and interphase cells from newborns with trisomy 21**

Nakamura et al. (2014) recently published their findings of telomere length in newborns with trisomies 18 and 21 being similar to newborns with diploid karyotypes. Limited and conflicting data exist as to whether individuals with trisomy 21 versus normal karyotype have shorter telomere lengths before birth or an accelerated rate loss after birth. We report the results of our small study on newborns with trisomy 21 that conflict with Nakamura's report.

Telomere FITC-labeled PNA probes (DAKO, Glostrup, Denmark) were hybridized to metaphases on deidentified slides from cultured peripheral blood from three newborns with trisomy 21 and seven newborns with diploid karyotypes. Digital fluorescence images of 20 metaphase cells per individual were captured to measure the overall length of chromosome 1 and of 1p and 1q telomere signals in micrometers. A ratio of combined telomere length to total chromosome length was calculated per individual. The average ratio of telomere to chromosome length for the controls was $0.094 \pm 0.027$ and for individuals with trisomy 21 was $0.070 \pm 0.017$. Telomere length in newborns with Down syndrome was significantly shorter than newborns with a normal karyotype using Student's t-test ($p < 0.05$). Telomere length was also examined in interphase cells for all chromosome arms by comparing the intensity of telomere PNA probe fluorescence to that of DAPI fluorescent stain. The average ratio of total telomere to total DNA fluorescence for control was $0.1136 \pm 0.057$ and for trisomy 21 was $0.0857 \pm 0.071$. The total telomere length for Down syndrome interphase cells was significantly shorter than for control interphase cells ($p < 0.01$). Our results suggest that the reduced lifespan of individuals with trisomy 21 is inherent at birth.

Telomere length in fetuses with Down syndrome has been reported to be shorter than in fetuses with normal karyotypes (Sukenik-Halevy et al., 2011). Since the pregnancies were terminated in this study, it is unknown whether these fetuses with trisomy 21 would have resulted in pregnancy loss or been liveborn, which may have been influenced by telomere length. However, Nakamura et al. (2014) recently reported that telomere lengths for newborns with trisomy 18 or 21 were similar to diploid newborns. Although our study of newborns involved fewer subjects, we found that telomere lengths of neonates with Down syndrome were statistically significantly shorter than newborns with normal karyotypes.

Telomeres, which shorten as an individual ages, are attributed to aging and age related diseases. Adults with trisomy 21 or Down syndrome have a lifespan of nearly 60 years of age compared to 78.5 years for the general population (Ebensen, 2010). Shorter telomeres have also been demonstrated to show an association with earlier onset of age-related degenerative diseases (Armanios et al., 2009) and Alzheimer disease (Panossian et al., 2003) than their normal cohorts, while individuals with Down syndrome and dementia have shorter telomeres than those with Down syndrome without dementia (Jenkins et al., 2008). Telomere loss in individuals with Down syndrome has been reported to occur at an accelerated rate (Vaziri et al., 1993). Our finding of shorter telomere lengths in newborns with trisomy 21 is consistent with the results of Sukenik-Halevy et al. (2011) reporting shorter telomeres in fetuses with Down syndrome. Another study examined telomere length in patients who were mosaic for trisomy 21, reporting a decreased telomere length in trisomic cells (Papavassiliou et al., 2007), suggesting that the telomere length is related to the extra chromosome 21. Our findings suggest that shorter telomere length in individuals with Down syndrome is an inherent trait, predisposing them to earlier onset of age-related diseases as well as shorter lifespan. Our results suggest that the reduced life span of individuals with Down syndrome is inherent at birth. Further studies are necessary to confirm these findings.

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