POSTERIOR HOMEOTIC TRANSFORMATION OF THE CERVICAL-THORACIC BORDER IS A MARKER OF MALDEVELOPMENT IN HUMANS

by

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STATEMENT OF THESIS APPROVAL

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ABSTRACT

This study was initiated after observing a high incidence of cervical ribs in stillborn fetuses referred for autopsy at our institution. The study establishes the prevalence of cervical ribs in this referral population and describes related associations. Radiologic data were reviewed from 389 stillborn and 171 liveborn autopsies performed at Primary Children's Medical Center from 2006 to 2011. Cervical ribs were identified in 49.1% of stillborn fetuses and 22.8% of liveborn infants at the time of autopsy. There was a statistically significant high association of cervical ribs in patients with aneuploidy. Karyotypes were available on 186 of the stillborn cases (47%). Of the patients with chromosome abnormality, 24 of 32 (75%) had cervical ribs. Our findings support the hypothesis that cervical ribs, a posterior homeotic transformation of the cervical-thoracic border, represent disadvantageous development during early stages of blastogenesis. This same region and patterning of the anterior to posterior skeletal axis has been conserved throughout evolution in almost all mammals.

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CHAPTER 1

INTRODUCTION

Background

This project was undertaken in an effort to determine the prevalence and to better define cytogenetic and molecular associations with the seemingly high incidence of cervical ribs identified in stillborn fetuses referred for autopsy at our institution. The high incidence of cervical ribs in stillborn fetuses compared to the incidence in adults, and the reported high incidence in children with cancers, prompted further investigation into the early development and organization of this area of anterior (rostral) to posterior (caudal) vertebral specification. The importance of this area in the evolution of mammalian morphological patterning and as a marker for maldevelopment manifested itself in the information gathered. The information suggests a potential tool for use in screening for childhood cancers that may be linked to the same developmental genes expressed in this area.

Animal Body Plans

The categorization of animal body plans is based on the origin and diversity of anatomical structures within phylogenetic groups. The "body plan" is a basic pattern of organization shared by a group of animals. This shared morphology originates during

development. Development of metazoans is the entire process by which a fertilized egg gradually gives rise to a multiorgan and multicellular adult organism. Specification of morphologic characteristics in a developing animal result from genetic regulatory programs set into place during blastogenesis. These developmental programs are set into place in the earliest stages of development beginning with the first few cells divisions forming the blastocoel. The inner cell mass, at one side of the sphere of trophoblast cells, gives rise to the embryo. At the beginning of mammalian gastrulation, the inner cell mass forms two layers: the hypoblast and epiblast. The hypoblast gives rise to the endoderm and the yolk sac. The yolk sac is a structure seemingly much more useful to our reptilian ancestors. The yolk is an essential nutrient source of animals that utilize yolky eggs. Because birds and mammals are descendants of reptiles, it is not surprising that their development is similar in early stages. The second cell layer is the epiblast, it gives rise to all three embryonic cell types including the ectoderm directly, then the endoderm and mesoderm through the primitive streak. As the developmental programs are set during blastogenesis, they will program specification of body plans.

The first mechanism proposed for the diversity arising during animal evolution was "natural selection" which utilized "descent with modification" or "transmutation" presented by Charles Darwin (1859). This model of natural selection allowed for inherited modification and change over time to select for the most successful (in reproductive terms) traits in a population. Darwin understood that these traits were inherited and passed to new generations through an apparent combination of phenotypes derived from two parental organisms. An important realization from his work was that all life on earth was descended from a common ancestor. The mechanism through which

these changes are made was not known at the time of Darwin. Gregor Mendel (1866) provided an insight into inheritance through his experiments with pea plants. The science of inheritance and transmission of diverse traits to new generations is termed Mendelian genetics. What Darwin and Mendel did not know at the time of their publications was that the genetic code for inheritance is provided in DNA.

One molecular mechanism of change and evolution is accomplished through recombination of DNA during meiosis. One recombined half of each parental DNA is supplied through their gametes and combined to form new and diversified progeny. Alterations in the sequence of chromosomes and genes can alter protein shape and function providing a route to changing the phenotype of an organism. One genetic alteration does not always affect a single phenotype as thought early in Mendelian genetics. Epistasis and pleiotropy are also inherent properties of biological systems. Epistasis refers to the interactions between genes in which one gene's contribution to a phenotype depends on the genotype at another locus. An example of this gene interaction is demonstrated with the human E4 allele of *ApoE*. This allele is associated with increased cholesterol levels, but only in individuals with an A_2A_2 genotype at the *LDLR* locus (Tyler, Asselbergs, Williams, & Moore, 2009). This and many other examples solidify the hypothesis that genes interact in complex networks and that disruption of one gene has implications over a wide spectrum of gene interactions (Tyler et al., 2009). Pleiotropy is defined as one mutation resulting in multiple, apparently independent phenotypes. Pleiotropic genes provide an example of the extensive network of not just one gene for one phenotype but thousands of genes interacting to produce a complex phenotype. Often, the same genes and proteins are used for signaling factors in multiple

pathways. In this manner, the single gene mutation of *Gli3* in the Pallister-Hall syndrome manifests itself in multiple phenotypes of many different developmental locations including polydactyly, digit webbing, central nervous system, and kidney abnormalities (Hall et al., 1980; Hill, Wang, & Ruther, 2007). Genes interacting with each other by location on paired chromosomes (cis or trans regulation) may effect regulatory changes in expression to provide yet another mechanism for diversification (Wittkopp, Haerum, & Clark, 2004).

Through evolution and diversification, the appearance of key developmental genes led to major transitions in animal body plans. These key developmental genes are present in even the most basic of metazoan species and many of these genes are ancient in origin (Srivastava et al., 2010). This concept is demonstrated in the chordate evolution from a common ancestor over 520 million years ago. A model organism to demonstrate this is *Branchiostoma floridae*, often referred to as amphioxus or a lancelet. The small amphioxus genome of 520 Mb has a basic set of chordate genes. These include a 15th *Hox* gene and all genes in a single dose. The neural crest is not present in amphioxus, yet it has all the transcription factors utilized in vertebrates for the neural crest regulatory network. Ectoderm markers, neural ectoderm markers, border specifiers, neural crest specifiers, and downstream mediators of neural crest migration and specification genes are all present in amphioxus. Therefore, neural crest development in vertebrates is not due to new regulatory genes but due to acquisition of new functions by pre-existing genes (Holland et al., 2008).

Many gene families are represented by single genes in amphioxus but by many different copies of the same gene in vertebrates. Vertebrates often have two, three, or

four copies of the same gene that have been derived from two whole-genome duplications (Putnam et al., 2008). The evidence to support whole genome duplication and proposed mechanisms were summarized by Furlong and Holland (2002). First, the gene number of invertebrates does not exceed 20,000, whereas vertebrates have many more genes. Additionally, vertebrates have four copies of homologous genes that are present as a single copy in invertebrates. The importance of looking at the phylogenetic relationships of animals when comparing genomes is stressed. This aids in comparing single gene duplications versus entire genome duplications and relative timing on an evolutionary scale. Most gene families analyzed show gene duplications in the vertebrate lineage after it had diverged from early chordates, such as the amphioxus, to animals with true bony vertebrae. The location of linked genes on segments of chromosomes provides evidence that the mechanism of two genome duplications was accomplished through two sequential autotetraploidy events. The timing of this gene duplication was at the base of the vertebrate lineage. The early development of key genes used in multiple pathways and functions along with whole genome duplications explain, in part, the sudden appearance of many diverse body plans during the rapid increase in the number of new species of the Cambrian period around 550 million years ago (Erwin, 2011).

Permanent evolutionary change of structure or "body plan" involves changes to the dynamics of the embryologic self-organizing units. Developmental fields are the self-organizing units of the embryo (Opitz, 2012). In order to have a lasting change in these developmental fields, the process must have certain characteristics. First, it must be epimorphically hierarchical. From early cleavage to gastrulation and through ontogeny, all related species pass through a "pharyngula" stage of similar morphology. The lowest

gene expression is found in the germ band stage, ancient gene expression at the pharyngula, and the most modern genes expressed at the earliest and latest stages of development. These gene networks are epigenetically complex, nonlinear, and reciprocally interactive. They must be spatially coordinated and ordered. This also requires the genes to be temporally synchronized. As diversification progresses, the changes to "body plans" will be constrained into a few common developmental paths. Ancient genes and pathways recycled for novel evolutionary functions, homologous structures of anatomy, and recurrence of genetic conditions in humans that are otherwise normal in related species (so called atavisms) emphasizes a basis of evolution and descent from a common ancestor.

Anterior-Posterior Segmentation

Many animals are segmentally organized. This aspect of the organization of body plans allows for variation not only of the number of segments but also diversification of the segments. In vertebrates, somites are the embryonic structures from which the axial segments are derived. Vertebrates are a diverse group of animals (~57,700 species) sharing characteristics of indeterminate cleavage, tripoblastic organization, bilateral symmetry, and a vertebral column (Dunn et al., 2008). Vertebrate body segments include cervical, thoracic, lumbar, sacral and caudal regions. During early embryogenesis, a paired dorsal midline ridge of tissue (the primitive streak) appears in the epiblast. The cells adjacent to and within the primitive streak ingress and are subducted through the area of the primitive node and form the mesoderm (Deschamps & van Nes, 2005). As cells migrate through the primitive node, they are specified to form paraxial mesoderm.

Paraxial mesoderm later forms the somites. Somites ultimately become the vertebrae, muscle, and dermis. Somites form in anterior to posterior order and in regular intervals. They occupy equal space per new somite and form at precise times. The somites are all the same size in individual species along the anterior-posterior axis. The amount of time it takes to form a somite is equal to and specific to each species. A comparison of select species demonstrates that chick embryos form a somite every 90 minutes, mice every 120 minutes, and zebrafish every 30 minutes. The total number of somites is characteristic of an individual species (ex: 52 in chick, 65 in mouse, and greater than 500 in some snakes).

The process of forming sequential somites has been shown to follow a “clock and wavefront” model. The "clock" portion of the mechanism includes oscillating components of the Wnt, Fgf, and Notch pathways (Aulehla et al., 2003; Aulehla et al., 2008; Dequeant et al., 2006). These oscillators are generated by delayed negative feedback between pathways. Components of the system alternate between active and inactive expression and are self-inhibitory. Transcriptional targets of each pathway act as negative regulators. These regulators are unstable and wax and wane during the clock oscillations. In vertebrates, oscillation of the Notch pathway is the most basic component of the segmentation clock. In amniotes, the Fgf and Wnt components also oscillate using this clock model. The other component of the model is the "wavefront". The entire vertebrate embryo has mutually antagonistic gradients (Dubrulle & Pourquie, 2004). Initially, Fgf8 is present at high concentrations in the posterior portion of the embryo. The Fgf gradient is created by mRNA decay. The high concentration at the tail bud is degraded by newly forming tissue. The new mRNA gradient is then converted to an Fgf8 protein gradient. Retinoic acid (RA) level is high in the anterior mesoderm. The RA

gradient is established by a constant source at the node which forms a gradient as the node regresses (Aulehla & Pourquie, 2010). It follows that Fgf8 prevents initiation of segmentation and RA relieves inhibition by antagonizing Fgf. Functional tests have shown that Fgf 8 beads placed in the caudal mesoderm lead to the formation of smaller somites. When RA production is blocked it also leads to smaller somites. Conversely, if Fgf 8 production is blocked it will increase the size of the somites. Also, when RA beads are placed in the caudal mesoderm, enlarged somites are formed. The wavefront regresses with each somite formed and progresses posteriorly. The anterior gradient border correlates with the somite boundary. The clock oscillations are terminated at the boundary to the newly formed somite. New sets of gene expression reset the next somite for initiation of the clock. This repeat of clock and wavefront processes is responsible for the anterior-posterior basis of vertebrate segmentation (Pourquie, 2001). Fgf8 is another excellent example of utilization of a single gene in many places and pathways during development. In situ hybridization of Fgf8 in 3-day-old chick embryos have demonstrated staining in somites, limb buds, branchial arches, midbrain to hindbrain border, eyes, and the tail bud developmental areas.

The *Hox* Genes

A notable exception to the genes of ancient origin is a novel set found in all bilaterians: the *Hox* genes. *Hox* genes are developmental regulatory genes which, when mutated, cause segment-specific transformations. Homeotic transformations, originally described by William Bateson (1894), are changes in the identity of one part of the body plan into "the likeness of another" (see Figure 1). These anterior-posterior segments have

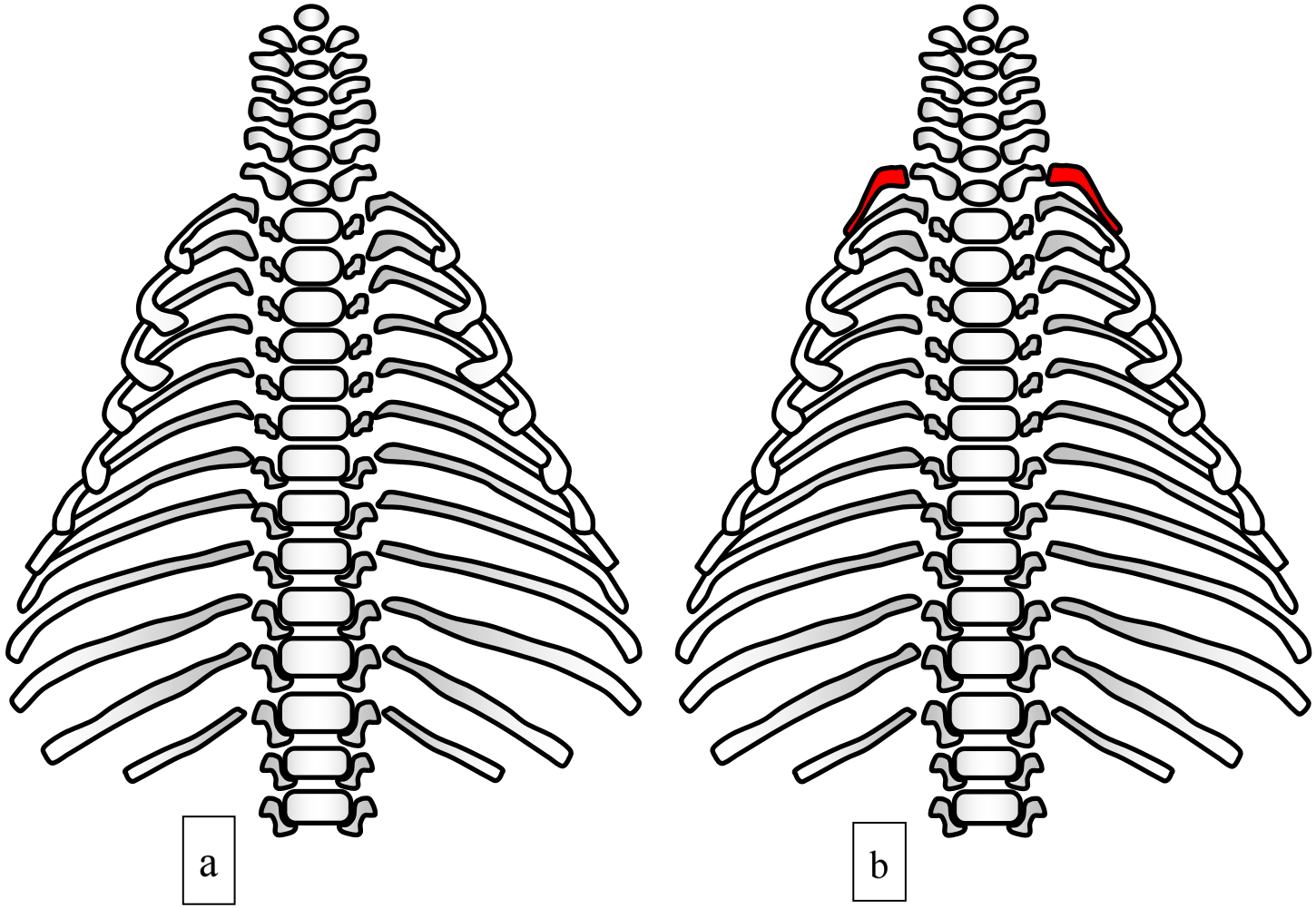

Figure 1: Posterior homeotic transformation of the cervical-thoracic border. a) Diagram of a normal approximately 25 week gestation human fetus. b) Homeotic transformation of the seventh cervical vertebra with an extra set of ribs on C7 (highlighted in red), taking on a thoracic-like identity.

overlapping *Hox* gene expression patterns. There exists clear evidence that mutations in the *Hox* genes will induce homeotic transformations in vertebrates (Wellik & Capecchi, 2003). The discovery of the *Hox* genes showed how these genes control the identities of structures along the anterior-posterior axis in the fruit fly *Drosophila melanogaster* (Lewis, 1978). Lewis postulated that fly segment identities were specified by unique combinations of *Hox* genes. These genes are not necessary for building any particular identity. Rather, they appear to select a particular identity among a number of possible identities. They act to program an identity at a specific time and place. When *Hox* genes were compared across species, an important and striking discovery revealed remarkable conservation between the homeodomains of these genes (McGinnis, Garber, Wirz, Kuroiwa, & Gehring, 1984). This conservation was seen not only in the amino acid sequence of these domains but also in the spatial order along the chromosomes. This spatial colinearity of the gene order in *Hox* clusters aligns with the body segmentations they specify. The *Hox* genes are shown to specify anterior-posterior segmentation in all bilaterians. Two genomic duplications early in vertebrate evolution have given rise to four *Hox* clusters in mammals (Dehal & Boore, 2005). The clusters are grouped into similar corresponding positions along the chromosome and maintain sequence similarity. These clusters of gene similarity and position are called paralogous groups. The 39 mammalian *Hox* genes are divided into 13 paralogous groups on 4 chromosomes. The genes are arranged 3' to 5' along the chromosome and are expressed at progressively later stages in development from anterior to posterior (Swalla, 2006) (see Figure 2). The roles of the *Hox* genes toward the 5' end of the chromosome that specify thoracic to caudal

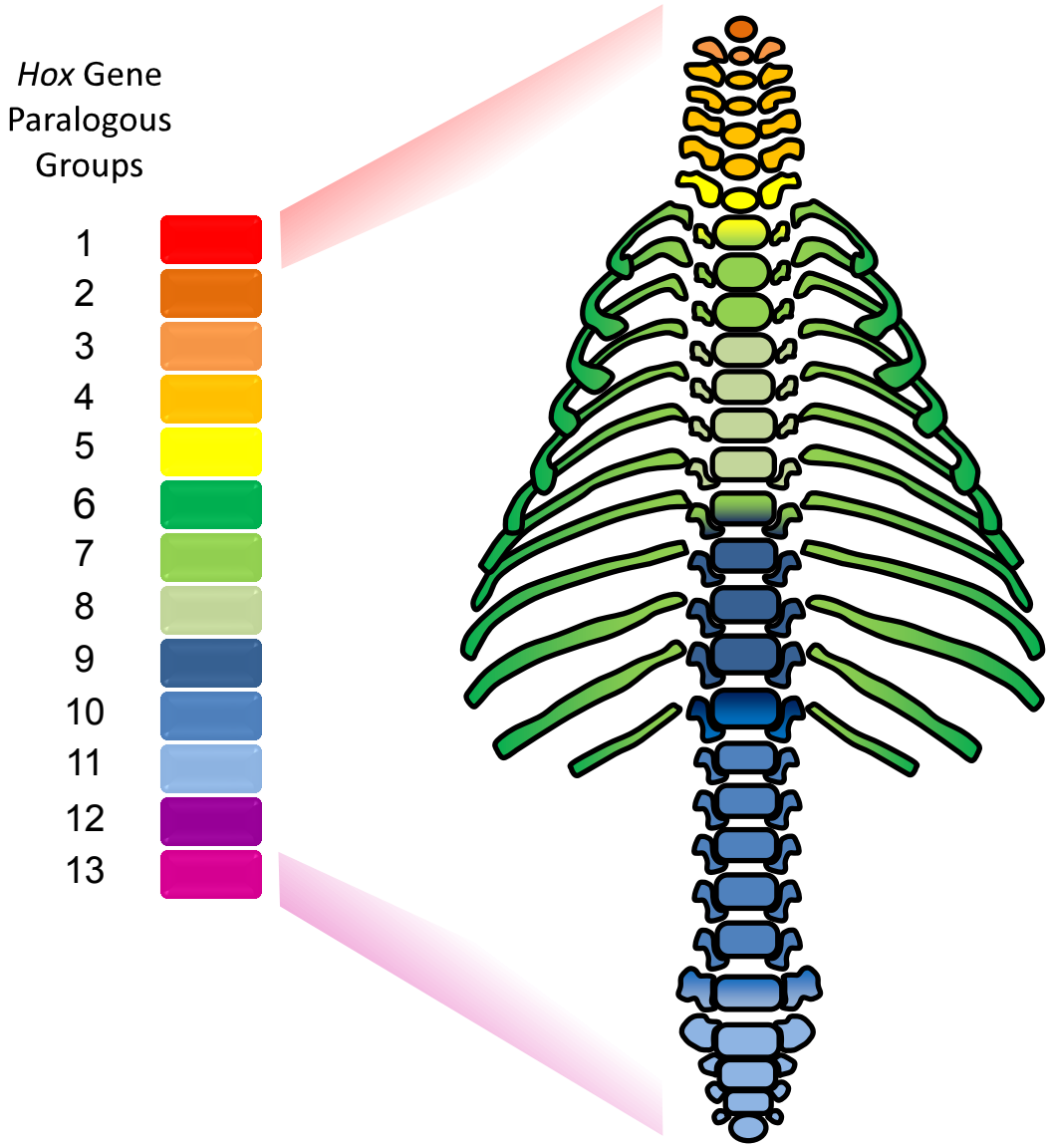

Figure 2: Proposed *Hox* gene expression in a human fetus based on mammalian animal models.

identity have been studied more extensively. Less is known regarding the mechanisms of specificity of cervical vertebrae and rib formation. *Hox* groups 3, 4, and 5 have a demonstrated role for patterning in the cervical skeleton (Horan et al., 1995). *Hox*a3 and *Hox*d3 specify the identity of the atlas (Condie & Capecchi, 1994). In transgenic mice, loss of three of the four group 4 genes resulted in transformation from posterior cervical vertebrae to first and second cervical identities (atlas and axis). Loss of group 5 genes resulted in defects in the cervical and thoracic segments (McIntyre et al., 2007). The complete change in the specificity of the cervical vertebrae into another identity has not been reported for any *Hox* mutants. The *Hox* paralogous groups 5 through 9 have been associated with development of the rib cage. The specification of whether the ribs are sternal (connecting to the sternum anteriorly) or whether they are floating ribs is under the control of the paralogous group 9 genes (McIntyre et al., 2007). Floating ribs are activated by this gene group expression and when the group is inactivated the ribs all connect to the sternum. There have been no reports of paralogous groups, which when deactivated, result in the complete loss of all ribs. This indicates that there is more than one group of genes required for rib formation. There could also be genes, not yet discovered, that play a role in the specificity of the rib cage. Defects in rib development have been reported for mouse mutants in the gene groups of *Hox*5 through *Hox*9. Gain-of-function experiments in which transgenic mice overexpress *Hox* group 6 genes have shown rib formation from vertebrae in the cervical and lumbar regions (Vinagre et al., 2010). This work suggests this gene group can specify thoracic identities to vertebral regions both anterior and posterior to the usual extent of this region. One of the first experiments demonstrating that *Hox* genes controlled segment identity along the axis of

the developing embryo involved *Hox*10 and *Hox*11 mutants (Wellik & Capecchi, 2003). When triple mutants were created from the three *Hox*10 gene paralogs in mice, a homeotic transformation occurred and lumbar to sacral segments were transformed into thoracic rib-bearing vertebrae. When the same experiment was performed with triply mutant *Hox*11 genes the sacral segments were transformed into lumbar vertebrae. Inactivation of all three genes in group 10 showed thoracic-like identity and rib-bearing vertebrae along the entire thoracic and lumbar area. The group 10 genes have rib-suppressing characteristics in mice. This was shown when ectopic overexpression of one of the *Hox*10 genes within this group resulted in complete absence of ribs. The group 10 genes have been reported to function in a manner opposite to that shown in mice when analyzing the elongation of the rib-bearing thoracic segment in the body plan of snakes (Di-Poi et al., 2010). In these animals, the expression domains of some members of the *Hox* group 10 genes extend into the thoracic segment and do not appear to repress rib growth in this area. This work also shows how transposon invasion of the *Hox* paralogous groups changed the sequence and apparent function of these genes. These changes of gene function are specific to squamates and partially explain the elongation to the body plan of these species.

The paralogous genes in the *Hox*11 group are essential for formation of the sacrum. When this group of genes was deleted in transgenic mice the sacrum was absent (Wellik & Capecchi, 2003). The *Hox*11 genes are known to function in fusion of vertebrae and adjacent structures to form the sacrum and iliac wings. The ectopic expression of *Hox*11 genes resulted in vertebrae fusing with a sacral-like identity. The genes in this paralogous group also are involved in the formation of the first two caudal

vertebrae. The genes of *Hox*12 and *Hox*13 appear to invoke a negative effect on elongation of the caudal vertebrae. Inactivation of *Hox* group 13 genes results in an increased number of caudal vertebrae. Activation under temporal control of these genes within this last grouping resulted in premature termination of axial growth (Young et al., 2009). The complete losses of some genes of group 12 and limited expression of multiple genes in group 13, have been postulated to cause the elongation of the snake body plan (Di-Poi et al., 2010).

The temporal and spatial expression of *Hox* genes control the axis development of the vertebrate body plan. Loss of function in one *Hox* gene allows for the expression of the more posterior gene resulting in the transformation of the segment. These genes code for targeted transcription factors that further define the segment identities. The effects of these genes would therefore depend on the unique characteristics of the proteins targeted by the individual *Hox* paralogous groups. Such is the case in the recent discovery of the mechanism of rib formation through the *Hox* group 6 rib-bearing segments along with the *Hox* group 10 rib-suppressing control (Vinagre et al., 2010). These genes modulate the expression of *Myf5* and *Myf6* in the lateral area of the myotome. The genes in group 6 promote this expression while the group 10 genes block this expression. The same mechanism is used when ectopic expression of these genes is induced to form the rib-bearing and rib-less areas in the embryo. When activation is modulated in the *Myf5* and *Myf6* genes, a similar phenotype of rib-less and rib-bearing areas is seen. The *Myf5* and *Myf6* genes seem to have redundant rib forming activity.

Another rib forming and activation mechanism was identified in the cervical vertebrae. Transgenic mice with overexpression of growth differentiation factor 11

(GDF11) were shown to have transformation of cervical into thoracic rib-bearing vertebrae (Li, Kawasumi, Zhao, Moisyadi, & Yang, 2010). Skeletal formation is mediated in part by the GDF11 activity. The anterior boundaries of the *Hoxa*-4 and *Hoxa*-5 expression domains were shifted toward the cervical region. In mice, homozygous null for GDF11, the embryos showed a more severe phenotype characterized by homeotic transformation of all segments along the axis as well as major malformations and decreased viability. Abnormal *Hoxa*-4 and *Hoxa*-5 gene expression with development of cervical ribs was also identified in the mouse deficient for the homolog of the *Drosophila polyhomeotic* gene (rae28) (Takihara et al., 1997).

Hox genes encode proteins with a conserved 60-amino-acid DNA-binding motif. They have been postulated to control expression of downstream genes in the diversification of body segments. The specific targets have proven difficult to identify because of the unique nature of these gene proteins (Hueber & Lohmann, 2008). The DNA binding of *Hox* proteins to their targets requires cofactors for specificity. Thus, these genes can regulate their own activity based on the availability of their cofactors. *Hox* genes can activate or suppress target binding depending on tissue components and developmental stage. The key to understanding *Hox* genes is identification of the target genes and analyzing their function in development. The targets and cofactors will then depend on the developmental stage of the organism.

Mammalian Segmentation Constraints

Large variation is seen within vertebrates of the total number of somites and also the number of somites per segment. Animals have evolved mechanisms for alterations in

Hox expression and specification leading to extremes of segments such as the 25 to 26 vertebrae of the neck of the swan, and over 300 thoracic vertebrae in some snakes (Gomez et al., 2008). One segment that has seemingly defied this variation is the number of cervical vertebrae of mammals. The number of cervical vertebrae is determined during the organogenesis stage in development. Animals categorized as mammals span approximately 5,500 species. All mammals studied to this point, except for some sloth species and manatees, have seven cervical vertebrae (Varela-Lasheras et al., 2011). The number of cervical vertebrae in mammals appears to have been under extreme evolutionary constraint. It would seemingly be beneficial for mammals such as giraffes to evolve more total cervical vertebrae, yet these animals maintain seven. Their vertebrae are enlarged and elongated but the number remains constrained as is seen in almost all mammalian species. This constraint on evolution mirrors phylogenetic constraints seen in the development of other organisms. Marsupials have extremely similar limbs across species. The size, musculature, and cartilage development is necessary for the fetus to grasp and crawl into the mothers pouch. Any variation that did not serve this need would be effectively selected against and eliminated from the gene pool.

Genes acquire new and diversified roles, often many times over, in developmental pathways and networks. If the functions are necessary in many essential pathways it would therefore be difficult to make lasting changes to the genes. Necessary genes then function with pleiotropic constraints because of their essential roles in different cells. Galis and colleagues proposed this as the mechanism for the conservation of segment polarity genes of insects (Galis, van Dooren, & Metz, 2002). Using the *Hox* genes of segment identity in vertebrates, she further postulates that a homeotic transformation of

the seventh cervical vertebrae to a thoracic identity and bearing ribs is selected against in mammals (Galis, 1999). The same *Hox* genes utilized in anterior to posterior axis specification are involved in stem cell proliferation in mammals. Mouse studies demonstrate that *Hox*a9 and *Hox*b4 can stimulate expansion of hematopoietic stem cells when overexpressed in these cells (Abramovich, Pineault, Ohta, & Humphries, 2005). Deletion of both copies of *Hox*a9 results in pancytopenia in mice (Lawrence et al., 2005). Thus, if changes are made to segmentation genes this may misregulate their effect in cell proliferation and lead to deleterious effects such as cancer. Aberrant *Hox* gene expression could contribute to oncogenesis by allowing activation of anti-apoptotic pathways (Shah & Sukumar, 2010). This has been demonstrated by rapid growth and increased size in recent evolutionary change. The high prevalence of central nervous system tumors in humans may be due to a recent evolutionary increase in brain size (almost threefold that of chimpanzees). Artificial selection in large dog breeds has produced an almost 180-fold increase in osteosarcomas as compared to smaller breeds. In humans, the hypothesis of negative selection toward a change in the number of cervical vertebrae is supported by findings of significant associations in patients with childhood cancers (Merks et al., 2005; Schumacher, Mai, & Gutjahr, 1992). Galis reports that children with cervical ribs have an extremely high chance of developing embryonal tumors (12%, a 120-fold increase compared to the general population) (Galis, 1999). These pleiotropic constraints are further evident in the reported instances of higher number of cervical ribs in stillborn fetuses (Furtado, Thaker, Erickson, Shirts, & Opitz, 2011). In a study of patients at autopsy in the Netherlands, Galis reported a rate of 54.8% of fetuses and infants with cervical ribs compared to a rate of 1.1% found in the highest

reported incidence in adults. She maintains that selection against pleiotropic effects is demonstrated by an increased cancer rate, associated congenital abnormalities, and stillbirth. She reported no statistically significant association between gender and age at death of stillborns with cervical ribs (Galis et al., 2006). The only mammals reported to have lost the selective constraint of seven cervical vertebrae are two-toed sloths and manatees that have six cervical vertebrae, and three-toed sloths that have up to nine. The proposed mechanism of relaxation of constraint is the low metabolic and activity rate severely reduces the stabilizing selection (Varela-Lasheras et al., 2011). Cervical ribs have also been reported highly prevalent in monosomy X (Keeling & Kjaer, 1999; Kjaer & Fischer Hansen, 1997). These constraints in development are likely due to a need for conservation of, not only the number of cervical vertebrae, but of the entire early organogenesis in mammals.

This study presents the prevalence of cervical ribs in stillborn fetuses at autopsy at our institution between 2006 and 2011. It was also conducted as a search for associations of cervical ribs with other biological variables. One variable specifically analyzed in our cohort was stillborn fetuses with aneuploidy. Traditional cytogenetic analysis can be challenging due to reduced numbers of viable cells and selective growth of maternal material sometimes present in samples derived from fetal tissue. Because the prevalence of cervical ribs has been reported increased in some aneuploidies, a determination of the accuracy of routine karyotype analysis was undertaken of patients with high suspicion of abnormal chromosomes based on the autopsy findings. Failed attempts of tissue growth necessary for routine karyotype were also included in the analysis by comparing these with a highly specific microarray on available archived tissue. This new microarray

platform allowed for the use of formalin-fixed paraffin embedded tissue that historically has not had success with other array platforms because of DNA degradation after formalin fixation.

CHAPTER 2

MATERIALS AND METHODS

Plain Films - Autopsy

The autopsy studies were approved by the Institutional Review Boards of the University of Utah and Primary Children's Medical Center. There were 389 stillborn fetuses (182 females and 207 males), ranging from 14 weeks gestational age to term. The liveborn control group consisted of 171 (68 females, 103 males) who died before 1 year of age.

A standard autopsy protocol was used for both stillborn fetuses and liveborn infants. Before an autopsy was performed, pertinent information from the medical record was reviewed. The external examination included anthropometrics, photographs, and whole-body radiography. Internal examination was limited to the extent of consent provided by the family on the autopsy permission form. Routine complete autopsies included removal, dissection, gross and microscopic examination of organs including measurements and weights. Tissue was sent for karyotype analysis on stillborns and liveborns with malformations or suspected syndromes. Chromosome analysis was also initiated at the request of the care providers. Radiographs were obtained in anterior-posterior and lateral positions using either conventional radiography (GE Healthcare,

adjusted to individual settings for optimal bone structure demonstration) or Faxitron (3 mA, 50-55 kV, 3-8 seconds, Hewlett-Packard Faxitron 43855A, McMinniville, OR, USA). The conventional radiography method utilized digital image storage directly into the institutional radiology image storage system. The Faxitron system employs a Konica digital radiographic cassette. The cassette can then be processed through the institution's scanning hardware to build the image for digital analysis. Faxitron images have provided optimal resolution for small fetuses (see Figure 3). The identification of lateral seventh cervical vertebral ossification centers and cervical ribs is accomplished through high resolution images in the anterior-posterior position of the neck. All bony processes off of the seventh cervical vertebral bodies identified beyond the lateral transverse processes of the first thoracic vertebrae were counted as cervical ribs (see Figure 4). When the cranium is positioned in slight dorsal flexion and the arms are by the sides of the body in the usual anatomic position with palms facing up, cervical ribs are displayed superior to the level of the sternoclavicular junction. The digital image analysis was done using IMPAX AGFA software version 6.3.1 that allowed user control of contrast, brightness, zooming, and optimal settings for the identification of even the smallest of cervical ribs (see Figure 5).

Analysis of Associations

A retrospective search of the pathology records of all cases from 2006 to 2010 was undertaken to search for associations of cervical ribs with congenital anomalies and aneuploidy. The anomalies were classified as either major or minor (Merks, van Karnebeek, Caron, & Hennekam, 2003). The anomalies categorized as major are

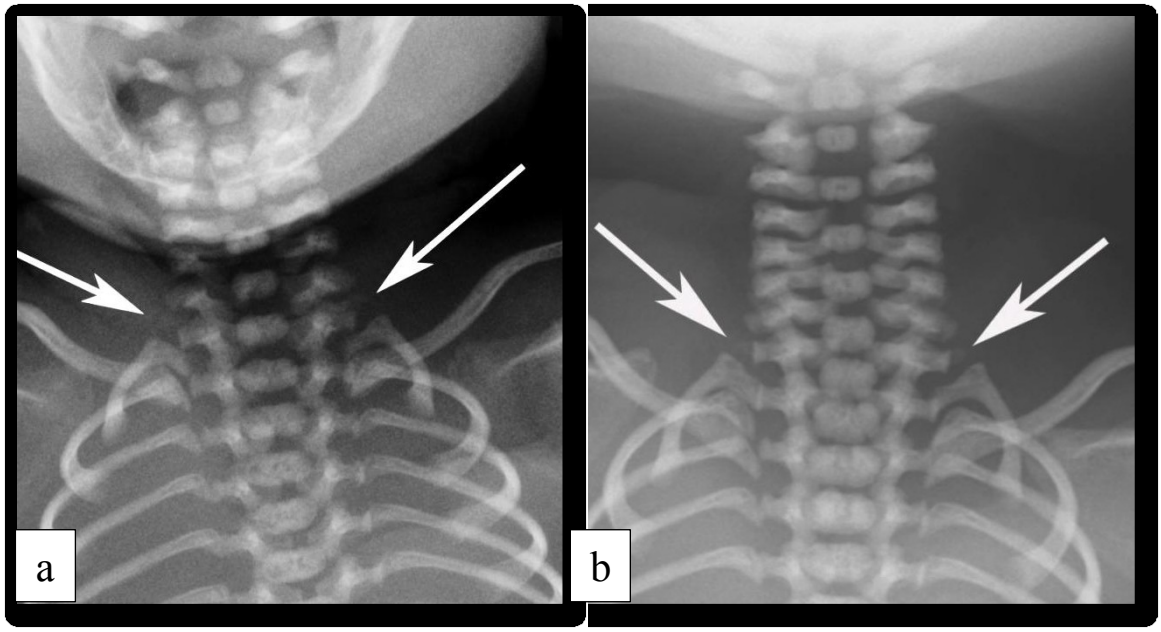

Figure 3: Comparison of conventional and Faxitron radiography in the same patient. a) Conventional radiography is hazy, blurred and difficult to see C7 bony detail (arrows). b) Faxitron radiography shows fine bony detail and just enough resolution to demonstrate bony extension of the C7 transverse processes (arrows).

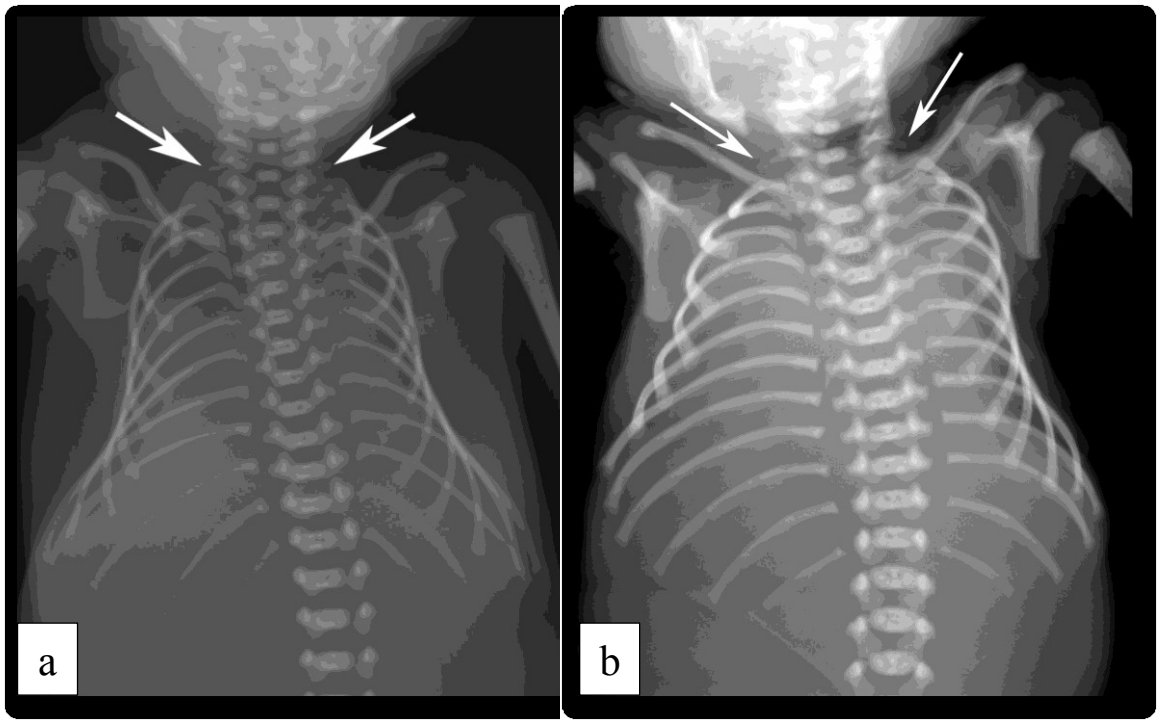

Figure 4: Images demonstrating cervical ribs of different sizes. a) Bilateral small ribs in a 23 week stillborn (arrows). b) Bilateral mid-sized cervical ribs in a 26 week stillborn (arrows).

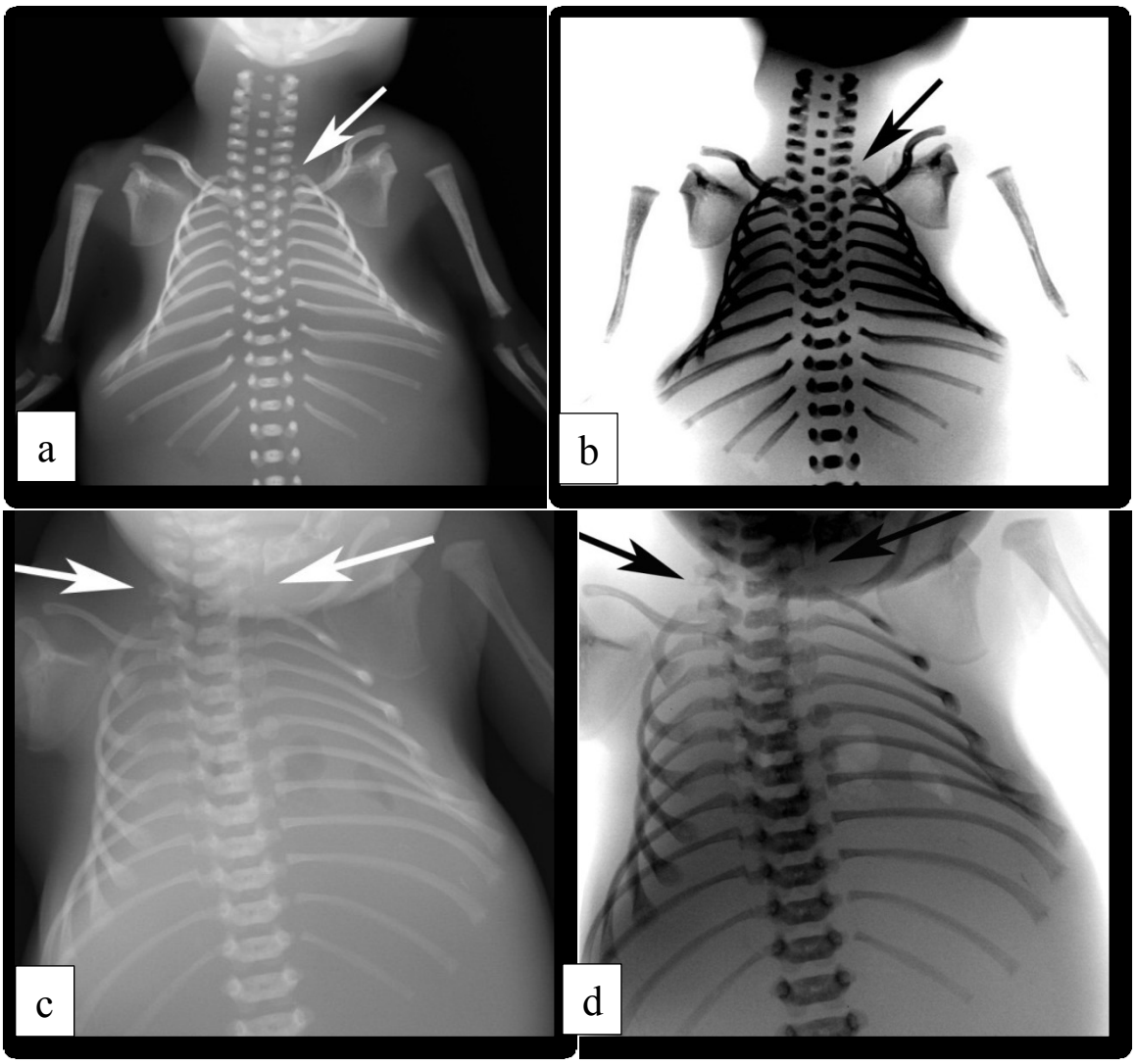

Figure 5: Software adjusted images to demonstrate difficult to see ribs. a) 20 wk fetus with unilateral cervical rib unadjusted (arrow). b) The same fetus as (a) with software adjustment for rib demonstration (arrow). c) 35 week fetus with soft tissue of the face obscuring the left cervical rib (arrow of patient's left side). d) image from frame (c) adjusted to demonstrate cervical ribs (arrows).

malformations that are defects of embryogenesis or other abnormalities including disruptions, dysplasias, deformations, and secondary to deficits of other structures. The major anomalies included: anencephaly, neural tube defects, cyclopia, cleft lip/palate, tracheoesophageal fistula, diaphragmatic hernia, renal agenesis, atresia of the aorta, interrupted aortic arch, and monoventricular/monoatrial heart. The anomalies categorized as minor are defects of phenogenesis. The minor anomalies included: ventricular septal defect, atrial septal defect, patent oval foramen, persistent patent arterial duct, omphalocele, retroesophageal right subclavian artery, hydrops, gonadal agenesis, accessory spleen, and anal atresia.

Analysis using the Chi-square model was used to determine whether the proportion of cervical ribs was different from expected compared to other skeletal anomalies, minor and major anomalies, chromosomal anomalies, and gender. The Fisher exact test was used to determine if there was any association between total number of anomalies (skeletal and/or congenital and cervical ribs). General linear modeling was used to determine whether the age of gestation was associated with cervical ribs in stillbirths.

Microarray Analysis of Suspect or Failed Karyotype

Traditional karyotype analysis for cytogenetic abnormalities has limitations. It can be challenging because of reduced number of viable cells. The viability of these cells is in question with cases of macerated fetuses even after only a short intrauterine retention time. There can also be selective growth of maternal cells sometimes present in samples derived from fetal tissue. Array comparative genomic hybridization (aCGH)

traditionally has required high quality DNA at high quantity. This has limited the use of formalin-fixed paraffin-embedded (FFPE) tissues for aCGH studies. We used a new technology array (OncoScan™, Affymetrix, Santa Clara, CA) to analyze FFPE tissue samples from archived autopsy tissue blocks in order to identify genetic abnormalities not previously detected using traditional cytogenetic methods. Formaldehyde can damage DNA and quality scores are low when used with traditional aCGH platforms. The high-density array probes used with the new array platform hybridize with FFPE tissues because of greater genomic coverage incorporating fine probe sequences.

Autopsy files were reviewed from 2007 to 2011 to identify cases in which the phenotype suggested underlying genomic alterations; the karyotype was either normal or not available and there were no other known genetic abnormalities; and previous microarray testing was not performed. Exclusion criteria included cases with known placental cause of death and those cases with extensive maceration. Controls included normal tissue from 6 samples used to generate the pooled average of normal intensities. Resulting copy number data were analyzed using Nexus v6.0 Copy Number software (Biodiscovery). Autopsy cases with known cervical ribs were included in the control samples and suspected abnormal cases. Paraffin scrolls were taken from archived autopsy blocks. In most cases, the liver and esophagus combined block was selected for use with the array. When liver tissue was not available, other tissues were also successful in producing valid results including: bone marrow, lung, pancreas, spleen, kidney, thymus, and adrenal gland.

CHAPTER 3

RESULTS

Postmortem Stillbirths and Infants

Cervical ribs were present in 191 of the 389 stillborns (49.1%) in this series. Of those, 111 were female (58.7%) and 80 were male (41.7%). Most cervical ribs were bilateral (76.4%). There was no significant difference between the numbers of single left-sided (51.1%) and single right-sided (48.9%) unilateral cervical ribs. In liveborns, subsequently analyzed at the time of autopsy, cervical ribs were identified in 22.8% of cases (39 of 171). Of the 39 cases identified with cervical ribs, 10 (25.6%) had unilateral ribs (see Tables 1 and 2).

The autopsy reports of 225 stillborns from 2006 to 2009 were evaluated for minor and major congenital anomalies other than cervical ribs. Cases with skeletal anomalies were reviewed. Skeletal lesions, other than cervical ribs, were seen in 20 (8.9%) of 225 stillborns. The stillborns with other skeletal anomalies also had cervical ribs in 12 cases. Although there was a limited number of cases within our series, there was no significant association with cervical ribs and other skeletal anomalies ($P = 0.11$).

Congenital anomalies, such as cleft lip/palate, tracheoesophageal fistula, diaphragmatic hernia, cardiac defects, omphalocele and fetal hydrops, were mentioned in

Table 1

Summary of cervical rib status in stillborns and liveborns

	Total	Cervical Ribs Present	Unilateral Ribs	Unilateral Left	Unilateral Right
Stillborns	389	191(49.1%)	45(23.5%)	23(51.1%)	22(49.9%)
Liveborns	171	39(22.8%)	10(25.6%)	4(40%)	6(60%)

Table 2

Summary of cervical rib status in stillborns and liveborns by gender

	Total	Cervical Ribs Present	Unilateral Ribs	Unilateral Left	Unilateral Right
Stillborn Males	207	71(34.3%)	20(28.2%)	11(55%)	9(45%)
Stillborn Females	182	96(52.7%)	25(26%)	12(48%)	13(52%)
Liveborn Males	103	19(18%)	4(21.1%)	3(75%)	1(25%)
Liveborn Females	68	20(29%)	6(30%)	1(17%)	5(83%)

the autopsy reports of 93 (41.3%) of the stillborn cases reviewed. Further statistical analysis of the data set did not show a significant association of congenital anomalies with cervical ribs ($P = 0.56$). The number of anomalies (congenital or skeletal) did not show a difference in those without cervical ribs.

Gender and gestational age were also compared with cervical ribs. Stillborn females were associated with cervical ribs in the series ($P = 0.003$). No association with gender was identified in stillborns with normal traditional chromosome analysis. Gestational age was not a statistically significant indicator of cervical ribs ($P = 0.90$).

In our patient sample there were two mothers that had multiple stillborns with cervical ribs. The first were both stillborn female fetuses (25 and 20 weeks gestation, respectively). Both of these stillborns had bilateral cervical ribs. The karyotype was available on only the 25 week fetus, which was normal. The second mother had female fetuses (both at 17 weeks gestational age), one with bilateral cervical ribs and the other with a unilateral right cervical rib. Karyotype testing was normal on both cases. There were no acute maternal conditions known to complicate these pregnancies.

Karyotype and Microarray Analysis

There were 186 chromosomal analysis results from the stillborn series. This number represented 47% of the total autopsied stillbirth fetuses. Cervical ribs were present in 76 of 154 (49%) of stillborns with normal chromosomes and 24 of 32 with aneuploidy (75%). Cervical ribs were present in 3 of 3 cases with trisomy 13, 1 of 2 with trisomy 18, 8 of 12 with trisomy 21, 9 of 10 with monosomy X, 1 of 1 with 46,XY,del(18)(q22), 1 of 1 with 48,XY,+2,+9/46,XY, 0 of 1 with 68,XX(with apparent

loss of one sex chromosome), 1 of 1 with tetrasomy 18p, and 0 of 1 with partial disomy of X (5.2 Mb duplication at Xq25-Xq26.2) (see Table 3).

Twenty-nine cases of fetal death were selected for a detailed analysis of the genome by microarray analysis. The storage time before analysis for the FFPE tissues ranged from one to 4 years. Samples from the study had 93.5% passing quality control scores. Genomic alterations were identified in 3 of 29 (10.3%) of the samples including deletion of 17q12 consistent with renal cysts and diabetes (RCAD), trisomy 18, and 4qter duplication with 13qter deletion arising from an apparently unbalanced t(4;13) translocation. No abnormalities were identified in the control samples (including the case with cervical ribs). The microarray also provided additional information regarding smaller (less than one megabase) gains or losses in 11 of the samples. Some of these areas included previously reported genes and phenotypes of known mutations. There were additional useful genomic data in 14 of 29 (48.3%) cases using this technology (see Table 4).

There were 11 cases in the microarray portion of the study that had cervical ribs. In 5 of the 11 cases (45.5%) additional information was identified. The areas of deletion, duplication or rearrangement did not match or overlap in any of the cases. The abnormal regions identified did not correspond to known *Hox* genes. Although helpful to the family and aiding in the completeness of the pathology report, none of the additional information proved useful in identifying areas of the genome for future focused cervical rib studies.

Table 3

Prevalence of cervical ribs in stillborns with abnormal chromosomes

	Total	Chromosome Abnormality	Cervical Rib(s) present
Stillborn - Normal Karyotype	154		76(49%)
Stillborn – Abnormal Chromosomes	32		24(75%)
		Monosomy X	9/10
		Trisomy 21	8/12
		Trisomy 18	1/2
		Trisomy 13	3/3
		46,XY,del(18)(q22)	1/1
		48,XY,+2,+9/46,XY	1/1
		tetrasomy 18p	1/1
		Partial disomy of X (5.2 Mb duplication at Xq25-Xq26.2)	0/1
		68,XX (loss of sex chromosome)	0/1

Table 4

Findings of microarray analysis

Patients (n=29)	Total	Microarray Result	Cervical Rib(s) present
Genomic Alterations	3(10.3%)		1(33.3%)
		Trisomy 18	0
		1.8 Mb deletion of 17q12	0
		unbalanced t(4;13) translocation	1
Additional Genomic Data Found	11(37.9%)		5(45.5%)
		265 kb gain of 3p21.1	1
		167 kb loss 2p16.3	1
		5kb gain 6p22.1 / 121 kb gain 6p22.1	0
		156 kb gain 18q12.1	0
		16 kb gain 13q33.1	0
		53 kb loss 9p24.1	1
		76 kb gain 1q42.3	0
		72 kb gain 3q13.31	0
		278 gain 1p34.1 / 243 kb gain 13q34	0
		535 kb gain 4q35.2	1
		566 kb gain 5q32	1

CHAPTER 4

DISCUSSION

Significance of Findings

Stillborn fetuses presenting for autopsy at our institution have a high prevalence of cervical ribs as compared to the liveborn control group. The prevalence as compared to the normal childhood population with cervical ribs (6.1% in children < 18 years) demonstrates an extreme marker of early mortality (Merks et al., 2005). It was reported previously that 78% of the fetuses with cervical ribs die before birth and 83% of liveborns before the age of 1 year (Galis et al., 2006).

It has been proposed that the stabilization of the number of cervical vertebrae across mammals is due to selective constraint. The selection has been reported as caused by deleterious pleiotropic effects (Galis & Metz, 2003). That is to say, that the genes (or changes in these genes) specifying this area of axis development and *Hox* gene expression are somehow promoting disadvantageous pathways leading to fetal death.

The second portion of the study demonstrated that the new higher density microarray platforms can add important data to the autopsy findings. Routine analysis in suspected cases may miss up to 10% of genomic alterations and up to 48% of potentially relevant chromosomal data. This information may be useful to a family planning for future pregnancies. It also has a potential for directing research at specific areas of the

genome when small deletions and duplications are identified. The study shows that results can be attained for FFPE tissues that have eluded other platforms. The fragmented and suboptimal DNA from formalin-fixed tissues will hybridize to the newer array technologies. Cases that have failed routine chromosome analysis can be utilized after many years of paraffin storage.

Limitations and Potential for Future Studies

The current study is limited due to the quality of the control group in the radiologic portion of the study. The cohort available for comparison was liveborn patients from the same autopsy series. Because these patients present at autopsy, they do not represent the findings of a more normal population of liveborns of the same age. There may be a higher prevalence of cervical ribs in liveborns who present at autopsy before the first year of life. These comparative images are not available due to the fact that chest radiography is not performed on healthy infants. These same limitations are found in previous studies that compare the rates in stillborn fetuses and infants at the time of autopsy to the reports of prevalence in adults. An ideal control group would include healthy newborns from various gestational ages. Bots and colleagues reported findings on the most normal cohort of early fetuses to date (Bots et al., 2011). They performed alizarin red staining on 199 electively aborted fetuses in a Finnish population. The unexpected result was 40% of the fetuses had cervical ribs. This result is comparable to our current study indicating the need for further analysis.

There is debate on the fate of the ossification centers that are identified on fetal radiography. Some authors have proposed that the lateral ossifications disappear after

postnatal life or are part of the lateral transverse processes of the seventh cervical vertebral body (O'Rahilly, Muller, & Meyer, 1990) (Chernoff & Rogers, 2004). Others have suggested that the ossification centers do not disappear and are in fact posterior homeotic transformations of the area. Specifically, the seventh cervical vertebra changes its characteristics to that of the first thoracic vertebra (Galis et al., 2006). A detailed study of the progression and fate of the ossification centers is needed. This would include serial images of multiple patients presenting early in postnatal life with evidence of different lengths and characteristics of cervical ossification centers (see Figure 4). Following these patients through to adulthood and completion of bone growth would allow definitive evidence of ossification center and patient fate. This may be possible in a retrospective analysis of patients requiring annual (or more often) chest films for routine care (e.g., for scoliosis or infectious diseases). Other imaging modalities should be included for additional detail (see Figure 6). Ultrasound, computed tomography, and magnetic resonance imagery will demonstrate detailed bony formation as well as associated soft tissue development (Hershkovitz, 2008).

This study presents an opportunity to look further into the pathways of associated genes in this area. A key to the understanding of the development of cervical ribs is the identification of the corresponding processes that specify their formation. The origin of the processes during organogenesis and development of cervical ribs is still uncertain. The understanding of how a homeotic transformation in such a highly conserved area of mammalian segmentation could predict intrauterine fetal demise continues to be an important area of focused research. The stresses to a developing fetus and the

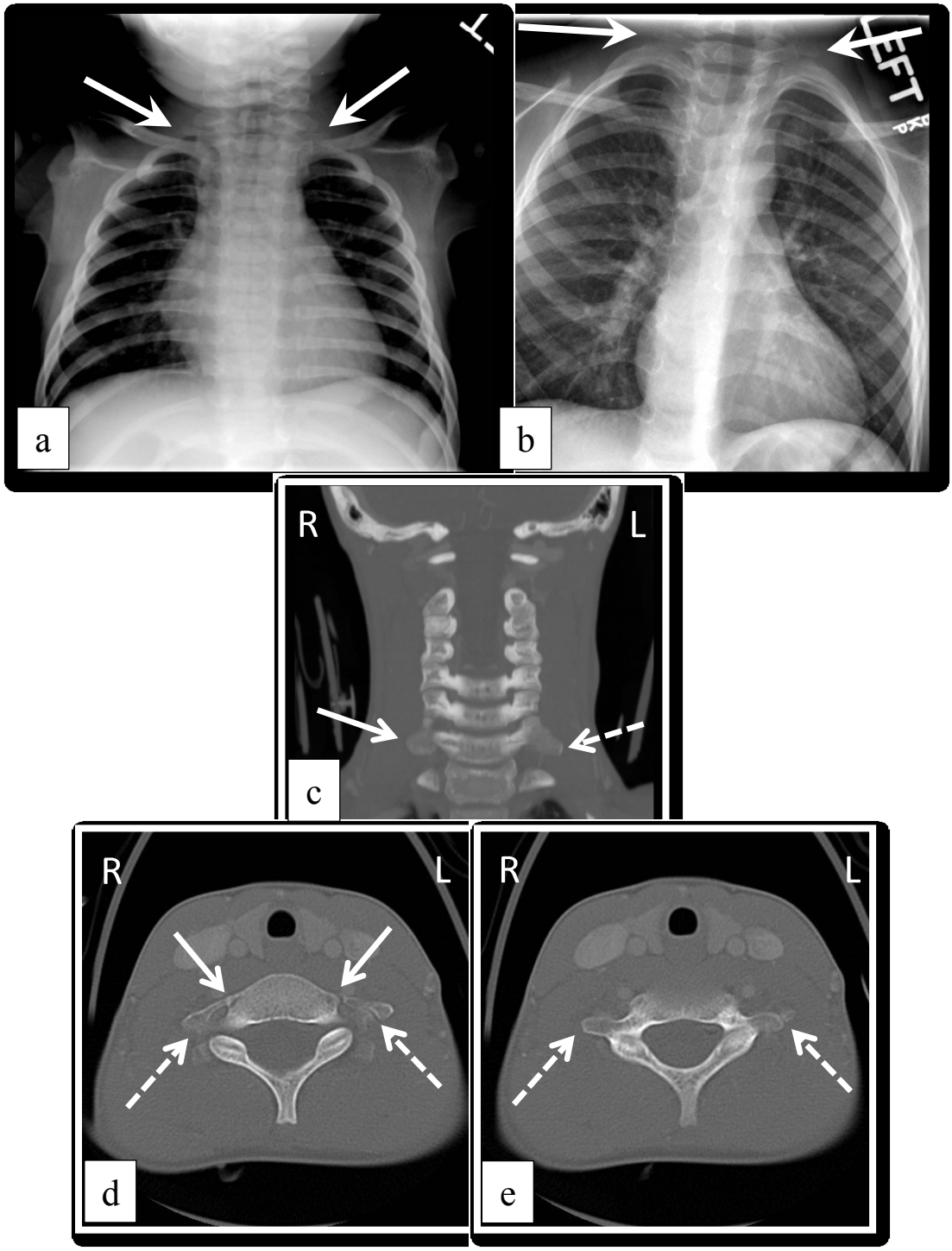

Figure 6: Example image types for a study needed to demonstrate fate of cervical ribs utilizing multiple imaging modalities. a) Three month old male with what appears to be rudimentary ribs at C7. b) Same patient at 5 years old, the relative appearance remains similar to that identified at 3 months. c) Coronal computed tomography image showing apparent bilateral extended transverse processes. d) & e) Axial images of C7 detailing that the right side has anterior (solid arrows) and posterior (dashed arrows) fusion of the transverse process, while the left shows a rudimentary rib without fusion.

mechanisms for increased mortality are not understood at this time. More directed studies into the genes and their pathways related to these areas are indicated.

Conclusion and Relevance to Medicine

Cervical ribs are not directly associated with a specific pathologic or anatomic diagnosis; however, a posterior homeotic transformation of the cervical-thoracic border in humans can be regarded as a marker of maldevelopment. This disadvantageous development is controlled by the *Hox* genes and their downstream targets. These genes have stabilized this area of the anterior-posterior axis developmental programs, as a rule, of all mammals except sloths and manatees. The stabilization of the seven cervical vertebrae in mammals has been presented on the basis of deleterious pleiotropic effects. Children found to have cervical ribs could be further screened for known associations. *Hox* gene expression affects the role of tumor suppression and oncogenesis (Shah & Sukumar, 2010). In leukemia, *Hox* gene overexpression promotes clonal expansion. In lymphoblastic leukemias with *MLL* translocations multiple *Hox* genes show increased expression and a worse prognosis including *Hox*a4, *Hox*a5, *Hox*a7, and *Hox*a9. These same clusters (a4 and a5) are the same genes involved with the specification of the cervical-thoracic border in humans. In neuroblastoma, expression of *Hox* genes will induce differentiation and prevent tumorigenesis. The mechanisms employed are threefold. The first, is the expression pattern of *Hox* genes is temporospatially different from that in normal tissue meaning that the expression may be turned on at different times and in different locations than seen in adjacent normal tissues. The second is gene dominance, as *Hox* genes are expressed at an increased level not normally seen in the

corresponding tissue type. The third, is silencing or downregulation of genes in tissues when normally they are expressed. Oncogenesis is associated with the temporospatial and gene dominance mechanisms while tumor suppression is evident with the downregulation mechanism. Cervical ribs can be used as a marker of disadvantageous expression of the *Hox* genes in this area. This has the potential as a screening indication for increased risk of childhood cancers if the associated networks are proven linked to the same processes.

Cervical ribs should be included in medical genetics evaluations because of the association with aneuploidy and abnormal chromosomes. Entire body radiographs in the anterior-posterior and lateral positions should be the rule for any postmortem pediatric autopsy. The identification of cervical ribs should be reported and the significance explained as part of the institutional pathology report following the autopsy. Pediatric radiologists should report on the presence or absence of cervical ribs in all cases.

Miscarriage and stillbirth are both risk factors for future second trimester miscarriages as well as for future stillbirth (Brigham, Conlon, & Farquharson, 1999; Heuser et al., 2010; VanderWielen, Zaleski, Cold, & McPherson, 2011). Finding the underlying cause of the homeotic transformation at the cervical-thoracic border may provide insight into the etiology of stillbirth. Human reproductive loss has continued at a high rate over many years (Silver, Branch, Goldenberg, Iams, & Klebanoff, 2011). Current practice and the understanding of the mechanisms of stillbirth have failed to decrease this rate. A greater understanding will mean earlier detection and enhanced predictors of outcome.

The higher density microarray should be utilized on tissues from stillborn fetuses with suspected genetic anomalies that have failed routine chromosome analysis. More complete information can be provided to the family in these cases. Areas of gain or loss may correspond to already known genes and phenotypes. This information may provide a more complete discussion with the family during genetic council sessions. Research gathered from the array analysis can direct future studies to provide necessary information to further the understanding of the etiologies of early human loss.

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