The Classification of VACTERL Association into 3 Groups According to the Limb Defect

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Summary: The VACTERL association (VA) is defined as the nonrandom co-occurrence of 6 anomalies: vertebral anomalies (V), Anal atresia (A), Cardiac defects (C), Tracheo-esophageal fistula (TE), Renal defects (R), and Limb anomalies (L). The current communication presents an argument that patients with VA should be classified into three district groups based on their limb defects: VACTERL1: patients with normal limbs; VACTERL2: patients with limb anomalies other than radial ray defects of the upper limbs; and VACTERL3: patients with radial ray defects of the upper limbs. The author will demonstrate that the rationale behind the L1-3 classification in patients in VA is based on the embryogenesis of the 6 affected anatomical areas in VA. The pathogenesis of VACTERL1 is secondary to perturbations of Sonic Hedgehog (SHH) interactions. SHH signaling is known to have a major role in the normal development of the vertebrae, ano-rectal area, heart, tracheo-esophageal area, and kidney. However, SHH is not involved in the development of the radial ray; hence, patients present with no limb defects. The pathogenesis of VACTERL2 is variable depending on the type of gene mutation. The pathogenesis of VACTERL3 is related to errors in a group of proteins (namely, the proteins of the TBX5-SALL4-SALL1 loop and the FGF8-FGF10 loop/ pathway). These proteins are essential for the normal development of the radial ray and they interact in the development of the other anatomical areas of VA including the heart and kidney. Hence, VACTERL3 patients present with radial ray deficiency. *(Plast Reconstr Surg Glob Open 2021;9:e3360; doi: 10.1097/GOX.0000000000003360; Published online 2 February 2021.)*

INTRODUCTION

The VACTERL association (VA) is defined as the nonrandom co-occurrence of 6 anomalies: vertebral anomalies (V), Anal atresia (A), Cardiac defects (C), Tracheo-esophageal fistula or esophageal atresia (TE), Renal defects (R), and Limb anomalies (L).[1,2] For a patient to be labeled as having VA, at least 3 of the 6 defects have to be present in the phenotype.[1,2] The literature explains the anatomical and functional rationale for these co-occurrences as a "related field defect." This indicates that the error has occurred at the same time of development of these 6 organs or that the gene/protein defect is responsible for the development of at least 3 of these organs. However, the literature completely overlooks that the pathogenesis of VA may be different for patients with normal limbs versus patients with limb defects.

The current communication presents an argument that patients with VA should be classified into 3 district groups based on their limb defects: VACTERL1: patients with normal limbs; VACTERL2: patients with limb anomalies other than radial ray defects of the upper limbs; and VACTERL3: patients with radial ray defects of the upper limbs. The author will demonstrate that the rationale behind this L1–3 classification is based on the embryogenesis of the upper limb and the development of the other 5 organs of the VA.

EPIDEMIOLOGY

The estimated frequency of VA ranges from 1 in 10,000 to 1 in 40,000 infants.[1,2] Series on VA do not specify the type of the limb defect. Hence, only the percentage of VACTERL1 versus VACTERL2/3 may be extracted from the literature. In most series of VA, about 50% of patients had no limb anomalies (ie, with VACTERL1) and the remaining 50% had limb anomalies.[3,4] However, the percentage of VACTERL1 patients was reported as high

as 76% of all VA patients in some series.[5] Although the literature describes patients with VA with limb versus no limb defects, no rational or perspectives were given to differentiate between these groups of patients. Hence, the main aim of the current communication is to introduce the classification of patients with VA into 3 groups according to the limb defect. This proposition is a new concept and is based on embryogenesis.[6]

VACTERL1 Patients

VACTERL1 patients have normal limbs but with involvement of the other 5 anatomical areas of VA. If our hypothesis is correct, then it would be expected that the key pathway responsible for this group of patients will mediate the development of these 5 anatomical sites, but not the development of the radial ray. The Sonic Hedgehog (SHH) signaling is known to have a major role in the normal development of the vertebrae (V), anorectal area (A), heart (C), tracheo-esophageal area (TE), and kidney (R).[7–16] Hence, it is of no surprise that there is a tendency for the co-occurrence of these 5 anomalies. In humans, loss of function of the *SHH* gene results in holoprosencephaly. Hence, the embryogenesis of VACTERL1 in humans is thought to be related to perturbations of SHH interactions rather than *SHH* mutations.[1] In fact, mutations in *HOXD13* (a down-stream target of SHH) and mutations/deletions of the *FOXF1* gene (also linked to SHH signaling) are known to cause a VACTERL1 phenotype in humans.[17–19] Furthermore, the ZIC3 protein is known to convert GLI3 (which is also related to SHH signaling) from the repressor to the activator form.[20] Hence, ZIC3 protein defects affect SHH signaling indirectly via GLI3.[20] *ZIC3* mutations in humans are known to cause a VACTERL–Hydrocephalous phenotype.[21,22] In contrast, SHH is not involved in the pathogenesis of radial ray deficiency.

VACTERL2 Patients

VATERL2 patients have a limb anomaly other than radial ray deficiency. Many patients with the VACTERL2 phenotype have mesoaxial or postaxial polydactyly secondary to either a *GLI3* mutation (such as Pallister-Hall Syndrome) or a ciliopathy syndrome.[23–25] Once again, GLI3 and ciliopathies are closely linked to SHH signaling (which is involved in the development of the first 5 anatomical areas of VA, as mentioned earlier). However, the VACTERL2 phenotype is associated with several other syndromes, and the pathogenesis is related to the function of the causative gene. Hence, it is important to note that VACTERL2 patients are not a coherent group and they present with different syndromes. The causative gene defect will affect the development of the first 5 anatomical areas of VA, and the same gene defect will result in a variable upper limb defect. Further examples of these various syndromes (other than the GLI3-related syndromes and the ciliopathy syndromes) will be given in the discussion.

The VACTERL3 as a Distinct Group

The VACTERL3 phenotype indicates the presence of a common pathogenesis resulting in both radial ray deficiency and VA. Hence, VACTERL3 patients should be considered as a distinct group. The degree of radial ray deficiency may vary from mild thumb hypoplasia to complete absence of the thumb and radius. Although the radial ray deficiency is the primary defect, few patients will also show concurrent preaxial polydactly.[26]

In the remaining part of this review, the author will reveal the common pathogenesis (via the TBX5 and FGF8 loops, as well as the FGF8 pathway) for both the radial ray defect and defects of the other 5 anatomical sites of VA in VACTERL3 patients. The role of these loops in the development of the radial ray will be reviewed first. This will be followed by a short review of the development of the other 5 anatomical areas of VA to demonstrate that the proteins of the same loops/pathways are also involved in the development of these anatomical areas.

The Normal Development of the Preaxial (Radial) Ray via the TBX5 and FGF8 Loops

Development of the radial ray requires complex ectodermal–mesodermal interactions on the anterior part of the limb bud.[27–30] Development of the radial ray requires 2 functioning loops: the TBX5-SALL4-SALL1-WNT loop and the FGF8-FGF10 loop (Fig. 1). The proteins of the former loop are expressed in the radial aspect of the mesoderm of upper limb bud, whereas the FGF8 protein is highly expressed in the radial aspect of the apical ectodermal ridge. Errors in TBX5 (T-BOX5) and SALL4&1 (Spalt proteins 4&1) result in radial ray deficiency as well as cardiac and renal defects because the proteins are also known to participate in the development of the heart and kidney. Similarly, errors of the proteins of the FGF8 pathway result in a VACTERL3 phenotype. Figure 1 shows the TBX5-SALL4-SALL1-WNT loop on the anterior mesoderm of the forelimb bud (the hindlimb bud has no TBX5 expression, and it expresses TBX4 instead). Reduction of Tbx5 in experimental animals results in down-regulation of Sall4, indicating that Tbx5 is involved in the expression of Sall4.[31] SALL4 and TBX5 are also known to interact to regulate the development of the heart and the radial ray in the forelimb bud.[32,33] The loop continues through SALL4–SALL1 interactions. SALL4 and SALL1 form heterodimers and both interact in anorectal, heart, and kidney development.[34] SALL1 will then enhance the WNT canonical signaling by localizing to heterochromation.[35] Finally, the enhanced WNT signaling activates SALL4 via the direct interaction of the lymphoid enhancer binding factor 1 (LFF 1)/T-cell factor (TCF) to the promotor of *SALL4*.[33] This loop explains the overlapping VACTERL3 features of Holt-Oram (*TBX5* mutations), Okihiro (*SALL4* mutations), and Townes-Brocks (*SALL1* mutations) syndromes.

Radial ray development also requires a high level of expression of FGF8 in the anterior aspect of the apical ectodermal ridge.[36] As shown in Figure 1, TBX5 is also involved in the second FGF10-FGF8 loop involved in the development of the preaxial ray. TBX5 induces and regulates mesodermal FGF10 expression.[37] FGF10 will then induce ectodermal WNT3A, which will induce ectodermal FGF8. The latter will participate in mesodermal FGF10 expression.[38,39] FGF8 is involved not only in the development of
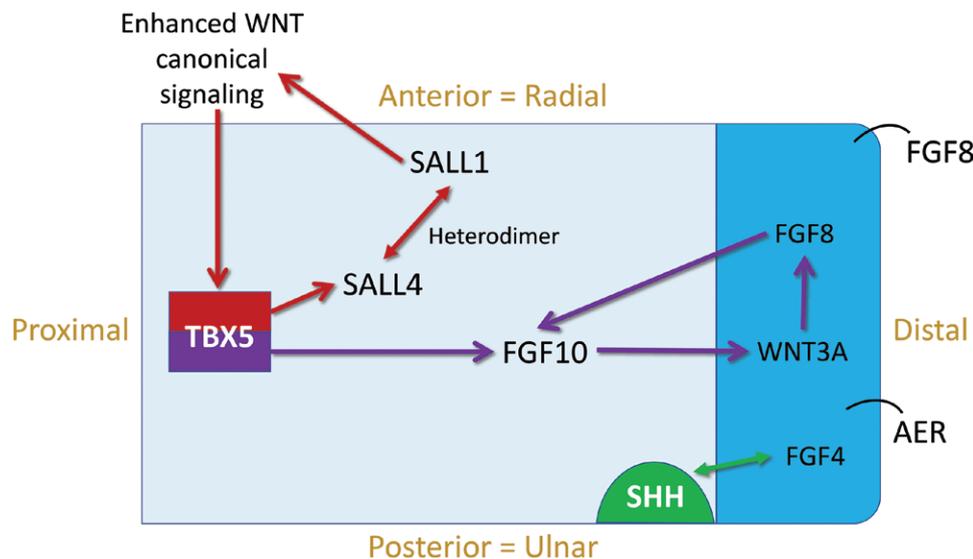
Fig. 1. Development of the radial ray requires the normal function of 2 loops on the anterior mesoderm of the forelimb bud: the TBX5-SALL4-SALL1-WNT and the FGF10-FGF8 loops. Note that TBX5 is a protein common for both loops; hence, it is double-colored in the figure. The first loop is colored red: TBX5 stimulates the expression of SALL4, and SALL4 makes a heterodimer (both proteins attach to each other) with SALL1. These 3 mesodermal proteins participate in the development of the radial ray of the upper limb and in the development of the heart and kidney. The loop is completed by the stimulation of WNT signaling (by SALL1), which in turn enhances TBX5 expression. The second loop is colored blue: TBX5 induces the expression of mesodermal FGF10, which enhances the expression of the ectodermal FGF8 (this occurs via WNT 3). The loop is completed by the enhanced expression of FGF10 by FGF8. The development of the ulnar ray requires the normal interaction between the ectodermal FGF4 and the underlying mesodermal SHH. AER, Apical Ectodermal Ridge.

the preaxial ray, but also in the development of the vertebrae (V),[40] heart (C),[41,42] Tracheo-esophageal area (TE),[43] and kidney(R).[44]

The FGF8 Pathway in the Upper Limb Bud and the Pathogenesis of VACTERL3

A brief description of the FGF8 pathway is essential to explain the pathogenesis of radial ray defects in the VACTERL3 phenotype. Ectodermal FGF8 diffuses to the underlying mesodermal cells on the anterior aspect of the limb bud. The main mesodermal receptor for FGF8 is FGFR1.[45,46] Upon stimulation of FGFR1 by FGF8, 3 pathways are activated: The Ras-ERK, the PI3K-AKT, and phospholipase C gamma-protein kinase C (PLC γ- PKC) pathways.[47,48] The former 2 pathways mediate cellular proliferation and differentiation. The interactions of these 2 pathways result in the induction of MKP3 (Mitogen activated protein Kinase Phosphatase 3).[47] Down-regulation of Mkp3 in experimental animals results in the induction of apoptosis in the mesenchyme.[47] The latter pathway results in a high level of PKC in the mesoderm.[48] Inhibition of mesodermal PKC in experimental animals results in limb truncation (similar to the effect of loss of FGF from the apical ectodermal ridge).[48]

The strength of FGF8 signal in the mesoderm is controlled by a double-step process of endocytosis.[46,49] The signaling strength of FGF8 in mesodermal cells is dependent on the rate of this endocytosis. In the first step, an invagination in the cell membrane occurs around the activated FGF8–FGFR1 complex. This results in the formation of a "vesicle" with a clathrin coat. The vesicle is then separated from the cell membrane by the enzyme dynamin. The vesicle (containing the FGF8–FGFR1 complex) fuses to the (Rab 5 + ve) early endosomes. In the second step, sorting of the FGF8-FGFR1 occurs to the (Rab 7 + ve) late endosomes for lysosomal degradation.[46,49] Two key regulators of endocytosis are the ubiquitin ligase Cbl[46] and the heat–shock cognate 70 (HSC-70).[49] The Cbl is an E3 ligase that acts as an adaptor protein during the endocytic activity, and its activity requires E1 and E2 ubiquitylation enzymes.[46,50] The HSC70 participates to disassemble the clathrin coat from the vesicle.[49]

Errors along this FGF8 pathway are expected to result in VA phenotype. A patient with a *FGF8* mutation was reported to have VA5. The PTEN (phosphatase and tensin homolog) is an important regulator of the PI3K–AKT pathway of cellular proliferation. One patient with *PTEN* mutation was reported to have a VACTERL3 phenotype (with hypoplastic thumbs).[51] Errors of ubiquitination may also result in a VACTERL3 phenotype. The Ephrin B2 (EPHB2) and the tumor necrosis factor–associated protein 1 (TRAP1) affect intracellular ubiquitination.[52,53] Several patients with a VACTERL3 phenotype were found to have deletions involving the *EPHB2* gene[54] or mutations in the *TRAP1* gene.[55] Similarly, HSC-70 participates in the regulation of the FGF8 pathway. Mutations in *HSPA6* are known to result in a VACTERL3 phenotype.[56] Both

HSC-70 and HSPA6 belong to the heat-shock protein 70 family, and both proteins have about 80% homology in strucure.[57]

Development of the Vertebral Column and the Role of WNT and FGF Signaling

The vertebral column develops from epithelial spheres called somites.[58] The dorsal parts of these somites form the skin and muscles of the back. The ventral parts develop into a mesenchymal sclerotome, which forms the vertebrae and ribs. The vertebral bodies develop under the influence of Pax1. FGF signaling also participates in vertebral development, including the formation of the vertebral ligaments.[58] WNT signaling is the main regulator of development of the cartilaginous endplates, which connect the vertebral bodies to the fibrous anulus fibrosis.[59]

Development of the Heart and the Role of TBX5, SALL4, SALL1, and FGF8

Initially, the primary heart field develops into the primitive heart tube (which is made of endocardial and myocardial cells). FGF8 has a role in the early differentiation of cells forming the primary tube.[60] Development of the chambers of the heart then occurs from further cell migration from the second heart field.[61] During this stage, components of the TBX5 loop (TBX5, SALL4, and SALL1) play a major role in heart development.[34,62]

Development of the Anorectal Area and the Role of WNT/FGF Signaling, and SALL4–SALL1 Interactions

The cloaca is the posterior digestive and urinary orifice of the developing embryo. This orifice is the future orifice of excretion of both urine and feces in amphibians, reptiles, birds, and a few mammals. In most mammals (including humans), the hindgut enters the cloaca and a separate anal orifice is eventually formed. WNT and FGF signaling regulate cloacal endodermal development.[63,64] SALL4–SALL1 interactions are essential in stem cell proliferation in the anorectal area.[34]

Separation of the Tracheo-esophageal Area and the Role of T-BOX Proteins and FGF

Initially, the trachea is adherent to the esophagus in utero. Many molecular events mediate the separation process. One process is through the normal development of the foregut folds.[65] Programmed cell death also plays a role in the separation process.[66] There are several proteins that mediate this process: SHH, NKX2.1, T-BOX, and FGF.[65] The study of these proteins is usually done in experimental animals such as the adriamycin mouse model of tracheo-esophageal fistula.[67]

Development of the Kidney, and the Role of SALL1, SALL4, WNT, and FGF

The urogenital ridge develops during the fourth week of intrauterine life. The nephrogenic cord of the ridge develops into the urinary tract. The fetal kidneys (the pronephros and the mesonephros) develop from the nephrogenic cord, but they degenerate later. The third kidney (the metanephric kidney) develops during the fifth week, and continues as the permanent kidney. Its development starts by the formation of the uretric bud, which branches to form the collecting tubules and the basic renal architecture. Functional nephrons will then develop from the collecting tubules. Initially the kidneys are located in the sacral area. At 6–9 weeks, the kidneys ascend to their normal lumbar location. The urinary bladder and ureters develop separately from the urogenital sinus.[68] There are many proteins mediating renal development, including SALL1, SALL4, and the WNT proteins.[34,69] FGF signaling is also essential in renal development. Experimentally, conditional deletion of FGF8 in nephron precursors interrupts nephron formation.[70]

DISCUSSION

The acronym VATER was introduced in 1972.[71] The letter "R" expressed the presence of both Renal defects and Radial (preaxial) dysplasia of the upper limb. Later, the acronym VACTERL was introduced to include any limb (L) defect in the phenotype.[1,2] However, radial dysplasia remains the most common limb defect in patients with VA and who have no identifiable syndrome.[3]

The current communication demonstrates that the development of the first 5 components of VA is influenced by SHH signaling, the TBX5-SALL1-SALL4-WNT loop, and FGF8 signaling. VACTERL1 is most likely related to errors of SHH signaling; and all VA cases with mutations of *HXOD13* and *FOXF1* (both related to SHH signaling) had a VACTERL1 phenotype.[17–19]

Patients with VACTERL2 usually have mesoaxial or postaxial polydactyly secondary to *GLI3* mutations or a ciliopathy syndrome[23], and GLI3 and cilia are also related to SHH signaling. However, the VACTERL2 phenotype is associated with several other syndromes (reviewed by Solomon[23]). The type of the limb defect (and other features of the syndrome) may also give a clue to the diagnosis of a specific syndrome. For example, patients with *WNT7A* mutations (Al-Awadi-Fuhrmann syndrome spectrum) are known to have a VACTERL2 phenotype. The WNT7A protein participtes in the development of the kidney[72] and the cardiac conduction system.[73] In the limbs, WNT7A participates in the dorsalization of the hand (the development of nails, extensor tendons, and the hairy dorsal skin), the development of the ulnar ray, and the outgrowth of the lower limbs. Hence, these patients present with duplication of the palms, ulnar ray deficiency, and truncation of the lower limb as their L3 phenotype.[72] Similarly, patients with ulnar-mammary syndrome (*TBX3* mutations) have cardiac and renal defects, but the clue to the diagnosis is usually made from the characteristic breast/apocrine gland hypoplasia and their characteristic ulnar ray defects (one-third of the patients have ulnar polydactyly, one-third have ulnar ray deficiency, and one-third have dorsalization of the little finger).[74]

Finally, the pathogenesis of VACTERL3 is through either the TBX5-SALL1-SALL4-WNT loop or the FGF8 pathway. It is important to realize that the pathogenesis of the VACTERL3 phenotype is still undetermined in some patients. For example, the Fanconi anemia genes are strongly expressed in the apical ectodermal ridge and the hematopoietic system explaining the 2 main features of the syndrome: radial ray
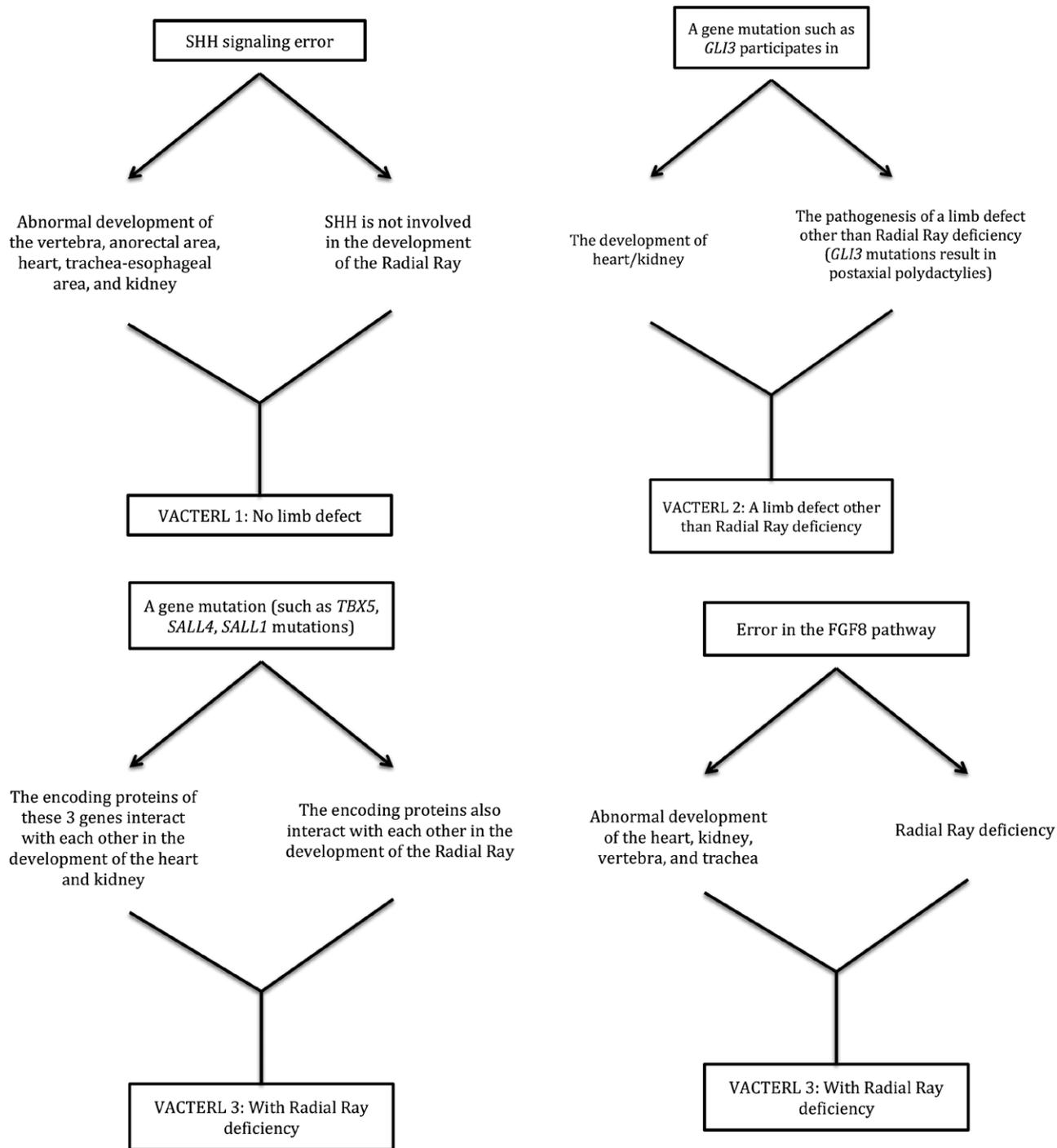
Fig. 2. Flow-charts summarizing the rationale behind the L1–3 classification in VACTERL patients: (1) VACTERL 1; (2) VACTERL 2; (3) VACTERL 3.

dysplasia and pancytopenia. Some patients with Fanconi anemia have renal and cardiac defects and hence, qualify for a VACTERL3 phenotype. The pathogenesis of radial ray deficiency in patients with Fanconi anemia is not fully understood, but it may be related to abnormal ubiquintin-ation[75] or secondary to the interactions of Fanconi anemia proteins with the WNT pathway.[76]

In conclusion, patients with VA should be classified according to their limb defects because the pathogenesis is different for each group. This concept is summarized in the flow-charts shown in Figure 2. This will also help as a guide for future research and the identification of candi-date genes for each group.[77]

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