Switch in FGFR 3 and 4 expression profile during human renal development may account for transient hypercalcemia in patients with Sotos syndrome due to 5q35 microdeletions.

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1   SWITCH IN FGFR 3 AND 4 EXPRESSION PROFILE DURING HUMAN RENAL DEVELOPMENT MAY

2   ACCOUNT FOR TRANSIENT HYPERCALCEMIA IN PATIENTS WITH SOTOS SYNDROME DUE TO 5q35

3   MICRODELETIONS

4

5   Running title: Ontogeny of FGFR 3 and 4 in human kidney

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29   **ABSTRACT**

30   CONTEXT: Sotos syndrome is a rare genetic disorder with a distinct phenotypic spectrum including

31   overgrowth and learning difficulties. Here we describe a new case of Sotos syndrome with a 5q35

32   microdeletion, affecting the fibroblast growth factor receptor 4 (*FGFR4*) gene, presenting with

33   infantile hypercalcemia.

34   OBJECTIVE: We strived to elucidate the evanescent nature of the observed hypercalcemia by studying

35   the ontogenesis of FGFR3 and FGFR4 – which are both associated with FGF23-mediated mineral

36   homeostasis – in the developing human kidney.

37   DESIGN: RT-qPCR and immunohistochemical analyses were used on archival human kidney samples

38   to investigate expression of the FGFR signaling pathway during renal development.

39   RESULTS: We demonstrated that renal gene and protein expression of both FGFRs increased during

40   fetal development between the gestational ages (GA) of 14-40 weeks. Yet, FGFR4 expression

41   increased more rapidly as compared to FGFR3 (slope: 0.047 vs. 0.0075, p = 0.0018). Moreover, gene

42   and protein expression of the essential FGFR co-receptor, Klotho, also increased with a significant

43   positive correlation between FGFR and Klotho mRNA expression during renal development.

44   Interestingly, we found that perinatal FGFR4 expression (GA 38-40 weeks) was 7-fold higher as

45   compared to FGFR3 (p=0.0035), while in adult kidney tissues, *FGFR4* gene expression level was

46   more than 2-fold lower compared to *FGFR3* (p=0.0029), thus identifying a molecular developmental

47   switch of FGFR isoforms.

48   CONCLUSION: We propose that the heterozygous *FGFR4* deletion, as observed in the Sotos syndrome

49   patient, leads to a compromised FGF23 signaling during infancy accounting for transient

50   hypercalcemia. These findings represent a novel and intriguing view on FGF23-mediated calcium

51   homeostasis.

52

INTRODUCTION

Sotos syndrome is an autosomal dominant disorder characterized by overgrowth, learning disability

and distinctive facial features such as macrocephaly [1,2]. The syndrome results from mutations and

deletions – mostly *de novo* – of the *NSD1* gene located at chromosome 5, which encodes for the

nuclear receptor-binding SET-domain containing protein 1 (NSD1). Little is known regarding the

function of NSD1 and it remains unclear how *NSD1* gene inactivation or NSD1 dysfunction may

cause the Sotos phenotype [3]. Intragenic mutations are responsible for 80-85% of Sotos syndrome

cases, while 5q35 microdeletions encompassing *NSD1* cause 10-15% of the cases in Europe and the

USA [4]. Of note, 5q35 microdeletions vary in size, from 0.4 to 5 Mb, and may affect genes flanking

NSD1 [5]. Saugier-Veber *et al*. mentioned the occurrence of nephrocalcinosis in three patients with

large deletions and suggested the presence of a predisposing gene in the deleted area [6]. This

phenotype spectrum was confirmed by Kenny *et al*. who reported two unrelated cases of Sotos

syndrome associated with nephrocalcinosis, one of whom had proven infantile hypercalcemia [7]. The

authors suggested that the heterozygous deletion of *SLC34A1* – encoding the main sodium phosphate

cotransporter NPT2a – could explain the temporary hypercalcemia in these patients. However,

hypercalcemia was absent in patients with a loss of function of NTP2a, as described by Magen *et al*.

[8], in contrast to Npt2$^{-/-}$ mice that exhibited a slight but significant increase in serum calcium

concentration compared to wild type mice [9]. Thus, the transient nature of the hypercalcemia remains

unexplained in Sotos syndrome patients.

Another gene mapped to the deletion interval is *FGFR4*, encoding for the fibroblast growth factor

receptor (FGFR) 4. The fibroblast growth factors (FGFs) are a large family of peptides that are

involved in a myriad of biological processes, including development and mineral homeostasis [10].

Currently, seven FGF subfamilies have been identified encompassing 22 different FGFs. The FGF19

subfamily – containing FGF19, FGF21 and FGF23 – plays a key role in regulating energy and mineral

metabolism [10]. Of interest, FGF23 is of the utmost importance in maintaining vitamin D, phosphate

and calcium levels [11]. FGF23 is a ligand for several FGFRs (*e.g.* FGFR1, FGFR3 and FGFR4) and

it has been demonstrated that Klotho forms a complex with these receptors resulting in an increased

affinity selectively to FGF23 [10,12]. FGFR1 is implicated in FGF23 effects on NaPi-2a and NaPi-2c

81 cotransporters [13], whereas FGFR3 and FGFR4 are linked to FGF23 effects on vitamin D and

82 calcium levels [14]. Thus, there is a clear link between FGFR4-mediated FGF23 signaling and calcium

83 homeostasis. Here, we describe a new Sotos syndrome case, due to a 5q35 microdeletion, with

84 infantile hypercalcemia and we hypothesized that a heterozygous mutation in *FGFR4* might be a

85 possible explanation for the observed elevated calcium levels. To unravel the temporary characteristics

86 of the perceived hypercalcemia, we studied FGFR3 and FGFR4 ontogenesis in the human developing

87 kidney that has never reported to date.

88

89 **MATERIALS AND METHODS**

90

91 *Ethics statement*

92 Fetal samples were collected after parental informed and written consent and after declaration to the

93 French Biomedical Agency (Decree 003812, 09/22/2006). Archival formol-fixed paraffin-embedded

94 fetal and neonatal kidneys were selected from the collections of several departments of pathology

95 according to the French legislation. Parental informed and written consents had been obtained at the

96 time of tissue collection and were conserved in each department. Experimental use of these samples

97 has been declared to the French Biomedical Agency (Decree 003812, 09/22/2006).

98

99 *Human renal samples*

100 Snap-frozen kidney samples from 18 fetuses, with a gestational age of 14–40 weeks, obtained from the

101 Fetopathology Department of Robert-Debré University Hospital (Paris, France), were used for qPCR.

102 For histology, a selection was made from thirty-seven archival tissue sections previously checked for

103 quality and integrity by immunostaining with vimentin and low molecular weight cytokeratin [15].

104

105 *Quantitative PCR*

106 To study gene expression, total RNA (1 µg) was reverse-transcribed using 50 U MultiScribe Reverse

107 Transcriptase (Life Technologies, Saint Aubin, France). Subsequently, cDNA was used for

108 quantitative PCR performed with a StepOnePlus Real-Time PCR system using the TaqMan Universal

109 PCR Master Mix (Life Technologies). GAPDH was used as housekeeping gene, and relative mRNA

110 expression levels were calculated using the equation: $(2^{-\Delta CT})*100$, or as fold change using the $2^{-\Delta\Delta CT}$

111 method. The following primer-probe sets were used: FGFR3, Hs00179829_m1; FGFR4,

112 Hs01106908_m1; GAPDH, Hs99999905_m1; Klotho, Hs00183100_m1 (all obtained from Life

113 Technologies).

114

115 *Immunohistochemistry*

116   Five-micrometer-thick tissue sections were deparaffinized and rehydrated in successive baths of

117   xylene and graded alcohols. Afterwards, the slides were heated in sodium citrate buffer (pH 6) at

118   100 °C for 15 min. Endogenous peroxidase was blocked with 3 % (v/v) H_2O_2 for 30 min. Next, non-

119   specific epitopes were blocked for 30 min at RT with 1% (w/v) bovine serum albumin in PBS

120   containing 0.1% (v/v) Tween-20. Subsequently, the slides were incubated overnight at 4°C with

121   rabbit-α-FGFR3 (1:1000; ab137084; Abcam, Cambridge, UK), rabbit-α-FGFR4 (1:500; ab41948;

122   Abcam) or rabbit monoclonal-α-Klotho antibody (1:100; ab181373; Abcam) in blocking buffer. To

123   reveal bound Ig, slides were incubated for 30 min at RT with the ImmPRESS anti-rabbit Ig kit

124   (Vector, Peterborough, UK), and liquid DAB plus chromogene (Dako, Glostrup, Denmark) was used

125   for visualization. Slides were counterstained with Mayer's hematoxylin and mounted using Glycergel

126   (Dako). Sections were studied via bright field microscopy (Olympus BX61) and images were taken by

127   means of a Retiga-2000R Fast 1394 digital camera (QImaging, Surrey, Canada).

128

129   *Statistics*

130   Statistics were performed using GraphPad Prism 6.03 *via* one-way analysis of variance (ANOVA)

131   followed by Bonferroni's Multiple Comparison Test or an unpaired Student's *t*-test. Differences

132   between groups were considered to be statistically significant when $p < 0.05$. The software was also

133   used to perform linear regression analyses and correlation analyses (Pearson and Spearman).

134

135 **RESULTS**

136 *Case report of a new Sotos syndrome patient with 5q35 microdeletion and infantile hypercalcemia*

137 G was the fourth child of non-consanguineous parents. His birth weight was 3580 g after 39 weeks of

138 uncomplicated pregnancy. A short period of artificial ventilation was required after birth due to

139 respiratory distress. Because of dysmorphic features, Comparative Genomic Hybridization (CGH)

140 array was performed, which revealed a microdeletion at 5q35, including both *NSD1* and *FGFR4*, as

141 shown in Fig. 1A. The deletion was absent in both parents, suggesting that it occurred *de novo*. In

142 addition, hypercalcemia was observed during the first two weeks of life, as presented in Fig. 1B, and

143 normalized afterwards. In the first weeks following birth from day 8-18, serum phosphate levels were

144 slightly reduced ranging from 1.22-2.54 mmol/L with a median value of 1.92 (n=8) (reference range:

145 1.74-2.66 mmol/L) and returned to normal concentrations afterwards. The maximum phosphate

146 reabsorption rate was decreased, possibly due to the heterozygous deletion of solute carrier family 34

147 (type II sodium/phosphate contransporter), member 1 gene (*SLC34A1)* also lost with the deletion (Fig.

148 1A) [16]. During the first 4 days, the child received intravenous nutrition and was then switched to

149 breastfeeding. At 4 years of age, he was more extensively investigated and it was demonstrated that

150 serum calcium and parathyroid hormone (PTH) levels were normal, whereas 1-25

151 dihydroxycholecalciferol (*i.e.* active vitamin D) was slightly elevated (86 ng/L, normal range: 20-80

152 ng/L). Nephrocalcinosis was absent on ultrasound scanning. We hypothesized that the heterozygous

153 deletion in *FGFR4* might be the possible explanation for the observed transient elevated calcium

154 levels.

155

156 *Expression profile of FGFRs during fetal renal development*

157 To elucidate the impact of the observed *FGFR4* deletion as well as the transient infantile

158 hypercalcemia, we studied the renal ontogenesis of FGFRs involved in calcium homeostasis. Fig. 2A

159 demonstrates that the gene expression levels of both *FGFR3* and *FGFR4* in kidney samples

160 significantly and positively correlate with gestational age (GA) with a calculated Pearson r of 0.63 (p

161 = 0.0054) and 0.72 (p = 0.0008), respectively. Moreover, the expression level of both receptors

162 steadily increases during renal development from 14 to 40 gestational weeks; yet there is a strikingly

7

163   greater increase in *FGFR4* gene expression as compared to *FGFR3* (slope: 0.047 *vs*. 0.0075, p =

164   0.0018). An analogous immunochemical pattern was observed with light microscopy, revealing a

165   similar amount of positively stained tubules at 21 weeks of gestation, while at a GA of 40 weeks,

166   FGFR4 protein seems to be more abundantly present as compared to FGFR3 (Fig. 2B). Furthermore,

167   in the fetal kidney, FGFR3 protein expression appears to be limited to the distal tubules, with some

168   stainings in the glomeruli as well as the medulla (Fig. 2B). In contrast, FGFR4 was detected in both

169   the proximal and distal tubules (especially at 40 gestational weeks; Fig. 2B) as well as the medulla

170   (Fig. 2B), while the receptor was absent in the glomeruli.

171

172   *Correlation between FGFR and Klotho expression*

173   It has been previously reported that Klotho is an essential cofactor for FGF23 signaling [10,17]. As

174   shown in Fig. 3A and 3B, Klotho mRNA and protein expression increases throughout renal

175   development, with low mRNA levels in fetal kidneys, consistent with the lack of detectable Klotho

176   protein in fetal and neonatal kidney samples, at variance with adult kidneys, in which Klotho protein is

177   readily detected in renal distal tubules. A significant positive correlation was observed between Klotho

178   mRNA and GA (calculated Pearson r = 0.80, p<0.0001). Moreover, the expression profile of Klotho

179   mRNA significantly correlates with both *FGFR3* (r = 0.6, p = 0.008, Fig. 3C) and *FGFR4* (r = 0.77, p

180   = 0.0002, Fig. 3D) as demonstrated with Spearman correlation analyses, consistent with the key role

181   of Klotho acting as a renal co-receptor for FGF23.

182

183   *Switch in FGFR expression profile during post-natal development*

184   Next, we compared the *FGFR* expression profile in fetal, neonatal and adult kidney samples. As

185   illustrated in Fig. 4A, renal *FGFR3* gene expression was extremely low in both the early and late stage

186   of gestation, with a significant 67-fold and 23-fold lower expression of the receptor as compared to the

187   adult level (p<0.0001). In contrast, renal *FGFR4* mRNA levels were only 5 times lower during early

188   gestation as compared to the adult situation (p = 0.004), while there was no significant difference in

189   renal *FGFR4* expression in the late stage of development as compared to adult tissue. Interestingly,

190   during late gestation, relative *FGFR4* expression is 7-fold higher than that of FGFR3 (p = 0.0035, Fig.

8

191   4B), whereas in adults, renal *FGFR3* expression level was significantly higher than that of *FGFR4* (p

192   = 0.0029, Fig. 4B). At the protein level, immunohistochemical studies revealed similar trend with

193   more tubules positively stained for FGFR4 than for FGFR3 at 2 and 9 months of age. Moreover,

194   FGFR3 and FGFR4 protein localization was similar in the neonatal kidney as compared to fetal

195   kidney. Conversely, FGFR3 protein expression was markedly increased in the adult kidney (60 years

196   old), and was detected in proximal and distal tubules as well as glomeruli, whereas there was a strong

197   decline in the number of FGFR4 positive tubules (Fig. 4C). These findings indicate a major molecular

198   switch in the respective contribution of both FGFRs in FGF23 signaling during human renal

199   development.

DISCUSSION

200

201 Sotos syndrome is a rare genetic disorder characterized by overgrowth, distinct facial features and

202 learning disabilities. The clinical features of Sotos syndrome vary between cases and seem to be

203 independent of genotype [3]. In addition, all features observed in microdeletion cases have also been

204 reported in individuals with intragenic mutations [3]. Still, the individuals with 5q35 microdeletions

205 are generally more likely to present with severe learning disabilities and less pronounced overgrowth

206 as compared to patients with intragenic mutations [18]. Furthermore, there is a nascent amount of

207 evidence that the phenotypic spectrum also encompasses impaired calcium homeostasis during

208 infancy.

209 Here, we report a new Sotos syndrome case, due to a 5q35 microdeletion, with evanescent

210 hypercalcemia. Since the deletion area included *FGFR4*, we hypothesized that dysfunction of this

211 FGF23 receptor might account for the altered calcium levels. Endocrine calcium homeostasis is an

212 intricate system involving several regulators and feedback loops. During hypocalcemia, PTH is

213 stimulated causing active vitamin D levels to rise, which in turn augments intestinal calcium

214 absorption as well as renal calcium retention [10]. In response to elevated serum calcium, osteocyte-

215 produced FGF23 is released. Binding of FGF23 to the FGFR-Klotho complex – including either

216 FGFR3 or FGFR4 – inhibits the synthesis of active 1,25 di(OH)-vitamin D3, both directly by reducing

217 1α-hydroxylase (CYP27B1) expression and increasing expression of the vitamin D degrading enzyme,

218 24-25 hydroxylase (CYP24) but also indirectly by decreasing PTH levels, resulting in a negative

219 calcium balance [10,17]. Thus, changes in FGF23 signaling will directly impact calcium homeostasis.

220 At present, both FGF23 pathway deficiency and renal FGFR ontogenesis have solely been studied in

221 animal models and human data is lacking. Therefore, we studied the *FGFR3* and *FGFR4* expression

222 profile in the developing human kidney.

223 Our results revealed a molecular switch in the FGFR expression profile during human renal

224 development. These findings provide compelling evidence that heterozygous FGFR4 inactivation in

225 humans, as observed in a 5q35 microdeletion Sotos syndrome case, might account for an impaired

226 FGF23 signaling pathway during early life causing transient infantile hypercalcemia. This notion is

227 corroborated by previous animal studies. For instance, Shimada *et al.* reported that calcium levels

228 were elevated in *Fgf23*^{-/-} mice [19], which is in accordance with the study by Yuan *et al*. [20], and an

229 analogous discovery was made in *Klotho* deficient mice [21]. Hypercalcemia was also observed in

230 *Ffg23* and *Klotho* double knockout mice [22]. Moreover, Haenzi *et al.* described that the loss of Memo

231 − a recently identified FGFR regulator − was associated with increased serum calcium [23]. Of

232 interest, the only phenotypic feature that has been reported in Sotos syndrome cases with

233 microdeletions is nephrocalcinosis [18]. Consistent with the transient hypercalcemia observed in the

234 described case, as well as those previously reported [7,18], it is likely that alterations of calcium

235 homeostasis might be a distinctive feature in Sotos syndrome associated with microdeletions.

236 Moreover, Tatton-Brown *et al.* performed a microsatellite analysis in 33 microdeletion cases and

237 detected a deletion of *FGFR4* in 32 individuals [5], strengthening the hypothesis that a mutation in

238 *FGFR4*, and subsequent impaired FGF23 signaling, is the culprit in infantile calcium imbalance.

239 Our study is one of the first to provide a detailed description of human renal FGFR ontogeny. Cancilla

240 *et al*., demonstrated that *FGFR3* and *FGFR4* gene expression could be detected in the developing rat

241 kidney and increased during development [24]. Furthermore, at embryonic day 20, FGFR3 protein

242 expression was present in the distal tubules, whereas immunostaining was absent in the proximal

243 tubules. In contrast, FGFR4 immunoreactivity was present in both distal and proximal tubules [24].

244 This expression pattern is similar to our observations. In addition, other studies have also

245 demonstrated the expression of *FGFR4* during renal development in rat and mice [25,26], and recently

246 it was reported in a zebrafish study that *fgf23* and *aklotho* were continuously expressed in the

247 developing kidney [27]. Several decades ago, Partanen *et al.,* reported the expression of both *FGFR3*

248 and *FGFR4* in human fetal kidney (GA 17-18 weeks) and they described that organ-specific *FGFR4*

249 expression highly differs from other FGFRs [28]. In a follow-up study, they investigated the

250 expression of FGFR4 in the developing mouse and, similar to our findings, they demonstrated that

251 *fgfr4* gene expression in the murine embryo steadily increased during development and was hardly

252 detectable in newborn and 2 day old mice [29]. Clearly, temporal FGFR expression is conserved

253 among species and is subjected to change during both early renal development as well as with aging.

254 The present study has some drawbacks; first of all, calcium homeostasis is a complex system

255 involving a myriad of negative feedback loops and multiple hormones [10], but unfortunately, during

256   the early clinical investigations serum levels of FGF23, soluble Klotho and active vitamin D have not

257   been determined. Secondly, we could not get access to mRNA from early postnatal kidneys, limiting

258   the latter part of our study solely to protein expression. Still, this study is the first to concisely describe

259   postnatal serum calcium levels in an individual with Sotos syndrome, and owing to the availability of

260   a unique and precious human kidney collection, we have been able to partially dissect and elucidate

261   the expression profile of FGFRs during human renal development.

262

263   In conclusion, we have demonstrated the presence of transient infantile hypercalcemia in a 5q35

264   microdeletion case of Sotos syndrome, which included a deletion of *FGFR4*. Furthermore, we found

265   that FGFR4 is highly expressed in fetal and neonatal kidney, whereas FGFR3 expression is highest in

266   adult tissue. This indicates that there is a developmental switch in the contribution of both FGFRs to

267   FGF23 signaling during aging, which could explain the fleeting hypercalcemia. These findings

268   provide a novel and intriguing insight in FGF23-mediated calcium homeostasis.

269

278

FIGURE LEGENDS
280

281 **Figure 1. New Sotos syndrome case with infantile hypercalcemia. (A)** Extent of 5q35

282 microdeletion including both *NSD1* and *FGFR4*. **(B)** Dots represent the calcium level per day during

283 the first weeks of life. Grey area depicts the normal range of total serum calcium in healthy neonates,

284 obtained from Roberton's textbook of neonatology (4th ed., 2005, Elsevier Churchill Livingstone).

285

286 **Figure 2. Expression of FGFRs in the human fetal kidney. (A)** *FGFR* gene expression was studied

287 using RT-qPCR. Relative expression was calculated as $(2^{-\Delta CT})*100$. Pearson correlation analysis

288 revealed a significant association between GA and *FGFR3* (O; r = 0.63, p = 0.0054) and *FGFR4* (■; r

289 = 0.72, p = 0.0008) gene expression. **(B)** Immunostaining of FGFRs in the developing kidney. **(C)**

290 Secondary antibody control for FGFR staining. Magnification, 20x. Arrows indicate a typical example

291 of a glomerulus (gl), proximal tubule (pt) and distal tubule (dt).

292

293 **Figure 3. Klotho expression in the developing kidney.** *Klotho* and *FGFR* gene expression were

294 studied using RT-qPCR. **(A)** Relative *Klotho* expression was calculated as $(2^{-\Delta CT})*100$. Pearson

295 correlation analysis revealed a significant association between GA (gestational age) and *Klotho* (r =

296 0.80, p < 0.0001). **(B)** Immunostaining of Klotho in fetal (GA of 40 weeks) and adult kidney tissue (60

297 years of age). Magnification, 20x. Arrows indicate a typical example of a glomerulus (gl), proximal

298 tubule (pt) and distal tubule (dt). **(C)** Positive correlation between *Klotho* and *FGFR3* (Spearman r =

299 = 0.6, p = 0.008). **(C)** Positive correlation between *Klotho* and *FGFR4* (Spearman = 0.77, p = 0.0002).

300

301 **Figure 4. Renal FGFR expression in fetal, neonatal and adult tissue.** *FGFR* gene expression was

302 studied using RT-qPCR. **(A)** Expression of *FGFR*s in fetal (early GA of 14-16 weeks; late GA of 38-

303 40 weeks) and adult renal tissues. Relative expression was calculated with the $2^{-\Delta\Delta CT}$ method. Data are

304 expressed as fold change relative to adult values. Statistical analysis was performed *via* one-way

305 ANOVA followed by Bonferroni's Multiple Comparison Test. **(B)** *FGFR4* expression in fetal (late,

306 GA of 38-40 weeks) and adult tissue as compared to *FGFR3*. Relative expression was calculated with

307 the $2^{-\Delta\Delta CT}$ method. Data are expressed as fold change relative to *FGFR3* levels. Statistical analysis was

308 performed *via* an unpaired Student's *t*-test. Results are presented as mean ± SEM of two independent

309 determinations performed in duplicate. *** = $p < 0.0005$, ** = $p < 0.008$, * = $p < 0.004$. **(C)**

310 Immunostaining of FGFRs in neonatal and adult kidney tissue (60 years of age). Magnification, 20x.

311 Arrows indicate a typical example of a glomerulus (gl), proximal tubule (pt) and distal tubule (dt).

312

313 **Supplemental Figure 1. FGFR expression in the renal medulla. (A)** Immunostaining of FGFRs in

314 fetal kidney tissue (GA of 40 weeks). **(B)** Secondary antibody control for FGFR staining.

315 Magnification, 20x.

316

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