Association of FSH receptor and CYP19A1 gene variations with sterility and ovarian hyperstimulation syndrome

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Abstract

Severe ovarian hyperstimulation syndrome (OHSS) is a potentially life-threatening complication during assisted reproduction technology (ART). The aetiology of this condition is still not fully understood. Several gene variations in the FSH receptor (FSHR) gene have been identified for the very rare cases of spontaneous OHSS. There are only few published data on gene variations in sterility and iatrogenic OHSS and no data regarding aromatase (cytochrome P450 19A1; CYP19A1). Ninety-one ART patients with OHSS, eighty-eight ART patients without OHSS and ninety-seven women with assumed normal fecundity were analysed for the FSHR single nucleotide polymorphism (SNP) gene variations Asn680Ser (rs6166), Ala189Val, Ile160Thr, Thr449Ile (rs28928870) and the CYP19A1 rs10046 locus using real-time PCR. In addition, exon 10 of FSHR of two patients with spontaneous hyperreactio luteinalis (HL) was sequenced.

Significantly lower frequencies of homozygous Ser680/Ser680 (P = 0.035) and heterozygous Thr160/Ile160 (P = 0.039) were found in patients with normal fecundity than those undergoing ART. The Ile160Thr SNP with a frequency of 6.7 and 6.1% in ART patients with and without OHSS respectively does not represent a rare mutation as previously published. There were no differences in the frequencies of all other gene variations. Of two patients with HL, both had homozygous point mutations for Ser680/Ser680 and one was heterozygous for Ile160Thr and CYP19A1 rs10046. The FSHR gene variations Asn680Ser as well as Ile160Thr may be contributing factors in unexplained sterility. The other FSHR coding gene variations and CYP19A1 rs10046 investigated are most likely not involved in the aetiology of iatrogenic OHSS or sterility.


Introduction

The World Health Organization (WHO) estimates that more than 80 million couples throughout the world are infertile (Hugues 2002). One in 10 couples is affected by primary or secondary sterility. Most of them live in developing countries, where the availability of assisted reproduction technology (ART) is very limited (Butler 2003). In contrast, women suffering from sterility in western countries are able to benefit from the achievements of reproductive medicine (Binder et al. 2004). Over the years, the number of centres utilizing ART throughout the world has increased and with it the number of stimulated cycles. One reason is that women are becoming pregnant at a later age (Butler 2003, O’Leary et al. 2007). The absolute numbers of patients who develop ovarian hyperstimulation syndrome (OHSS) are constantly rising in parallel with the increasing demand of artificial reproductive methods.

The condition of OHSS is potentially life threatening and mostly occurs during controlled ovarian stimulation. This syndrome is characterized by enlarged and polycystic ovari, ascites and generalized oedema, in addition to other symptoms (Delvigne & Rozenberg 2003, Binder et al. 2004). OHSS almost always occurs along with human chorionic gonadotrophin (hCG) therapy, used for the induction of ovulation, compounded by embryonic hCG in case of pregnancy. An excessive ovarian reaction to hCG leads to the development of more or less marked symptoms. Despite multiple published analyses, the aetiology of OHSS is not yet fully understood and several genes may be involved. The follicle-stimulating hormone receptor gene (FSHR gene; Fig. 1) appears to be one candidate for the very rare condition of spontaneous OHSS (Smits et al. 2003). One specific gene variation in the FSHR gene, which leads to a non-synonymous amino acid exchange at Asp567Asn, has been found to result in a conformation change of the serpentine region (Smits et al. 2003). This FSHR mutation appeared to cause a reduction in ligand specificity that allowed activation of the mutated receptor by hCG. In other patients with spontaneous OHSS, new FSHR conformation-altering mutations were found at codon 449, resulting in a substitution of threonine for either isoleucine or alanine.
(Thr449Ile and Thr449Ala; Fig. 1; Vasseur et al. 2003, Montanelli et al. 2004).

**Hyperreactio luteinalis** (HL) during spontaneously conceived pregnancies is a further interesting condition, presumably closely linked to spontaneous OHSS. This condition is also known to be associated with hyperandrogenism and polycystic ovaries (PCO). Typical manifestations are bilateral enlarged ovaries with multiple luteal cysts in early or late pregnancy but mostly without severe symptoms or ascites. There are considerations to assume that HL and OHSS could be entities in continuum (Haimov-Kochman et al. 2004). Some kind of inheritance seems to be also plausible, since in spontaneous OHSS several FSHR gene variants could be demonstrated, but to date no gene variations have been reported in patients suffering from HL.

Other FSHR gene variations causing more or less pronounced loss of FSHR function and affecting fertility have been published, such as the very common polymorphism Asn680Ser being in linkage disequilibrium with Thr307Ala (Al-Hendy et al. 2000, d’Alva et al. 2005) and rare mutations like Ile160Thr and Ala189Val (Fig. 1). Greb et al. (2005) investigated the influence of the FSHR Asn680Ser genotype on menstrual cycle dynamics in women and found differences in hormonal levels during the luteal phase. Aittomaki et al. (1995) and Sipila & Aula (2002) noted that all patients out of six families with autosomal recessive premature ovarian failure in Finland had an Ala189Val mutation in the FSHR gene. All these families were living in a small area with a presumably high rate of inbreeding. Beau et al. (1998) diagnosed other FSHR gene variations, Ile160Thr in combination with Arg573Cys, both heterozygous causing a loss of FSHR function in a 30-year-old Armenian woman who had already developed secondary amenorrhoea at the age of 16.

There are data showing that the outcome of controlled ovarian hyperstimulation may depend on interacting genetic and non-genetic factors that are related to hormone receptor activation. In mice the ovulation rate has been associated with increased cytochrome P450 19A1 (CYP19A1) aromatase gene activity and oestrogen production (de Castro et al. 2005b). In addition, there is evidence that the reference SNP ID 10046 (rs10046) single nucleotide polymorphism (SNP) locus of the aromatase (CYP19A1) gene in the 3’ UTR is associated with altered pituitary suppression in premenopausal women (de Castro et al. 2005a). This may suggest the hypothesis that gene variations activating the CYP19A1 gene may also be associated with OHSS.

The rationale behind this investigation was the assumption that activating SNPs and SNPs that could compromise FSHR function may show different distribution patterns in ART patients with or without OHSS compared with patients with normal fecundity and with HL. We therefore assessed the distribution of the following FSHR gene variations: Asn680Ser, Ile160Thr, Ala189Val, Thr449Ile and CYP19A1 rs10046 polymorphism in ART patients with or without OHSS, non-ART patients, and additionally sequenced exon 10 of FSHR in two patients with the rare condition of HL.

**Results**

**Distribution and morbidity of OHSS patients**

The mean age of the patients with OHSS was 34.80 ± 4.10 years, of the control groups 34.18 ± 4.27 (controls with ART/no OHSS) and 38.16 ± 5.60 (controls ‘no ART’). Additional clinical data were recorded for 86 patients with iatrogenic OHSS. The mean hospitalization period for these patients was 10.2 ± 6.5 days. Sixteen patients were...
suffering from grade I OHSS, thirty-one from grade II OHSS and thirty-nine from grade III OHSS. Sixty-one patients had early onset OHSS and twenty-five had late-onset OHSS. In those centres referring patients to our hospital, the incidence of severe OHSS was 0.7–1.2% and for moderate OHSS 4.0–6.0%. Mild OHSS was not assessed. Thirty-nine patients were pregnant, with an overall pregnancy rate of 45.3%. Fourteen patients had twin pregnancies, confirmed by ultrasound. Due to complications, three pregnancies were aborted and nine ended in spontaneous abortions. Twenty-seven OHSS patients delivered healthy babies, six by caesarean section and 10 before the 38th week of gestation. The baby take-home rate was 31.4%.

In the control group 73 patients out of the 88 (82.9%) had at least one recorded pregnancy or were pregnant when blood samples were taken.

Most OHSS patients had the following symptoms, in decreasing order of frequency (ICD codes in brackets): ascites (R18), pain (R10.1), increase in abdominal girth (R19), temperature $\geq 37^\circ C$ (R50.9), abdominal tension (R19.8), dyspnoea (R06.88), pleural effusions, nausea (R11), emesis (R11), generalized oedema (R60.1), diarrhoea (K58.0), urinary tract infection (N39.0), circulatory collapse (R55) and thrombosis (I80.2, I82.8; Fig. 2).

**Genotype frequencies for FSHR gene variations and CYP19A1 rs10046**

*FSHR* genotype analysis for Asn680Ser, Ile160Thr, Ala189Val, Thr449Ile and CYP19 rs10046 was carried out in a total of 278 women (91 with one or more recorded case of OHSS in previous ART cycles, 88 with no OHSS, 97 women with normal fecundity (Table 1) and 2 with HL). In the latter two, exon 10 was sequenced additionally (Table 2).

The distribution pattern for the rare gene variations of Ala189Val and Thr449Ile in the cohorts analysed were similar. Of all patients, 96.5–99% (‘OHSS’, ‘ART/no OHSS’ and ‘no ART’) were homozygous for Ala$^{189}$/Ala$^{189}$ (wild-type). A total of six patients (OHSS and controls) were heterozygous and none had a homozygous mutation. Evaluation of the Thr449Ile gene variation revealed that all analysed women were homozygous for the wild-type form Thr$^{449}$/Thr$^{449}$ (Table 1).

The CYP19A1 rs10046 genotype frequencies for cytosine/cytosine (C/C), cytosine/thymidine (C/T) and T/T were 22.0, 63.7 and 14.3% for the OHSS patients; 23.5, 57.6 and 18.8%, and 19.6, 59.8 and 20.6% for the control groups (‘ART/no OHSS’ and ‘no ART’) respectively. There were no significant differences between the groups (Table 1).

In the ‘OHSS’ group, 15.4% out of the 91 women were found to be homozygous for the wild-type Asn$^{680}$, while 51.6% were heterozygous (Asn$^{680}$/Ser$^{680}$) and 33.0% were homozygous for mutant Ser$^{680}$/Ser$^{680}$. The ‘ART/no OHSS’ control group revealed a distribution of 17.4 (wild-type), 46.5 (heterozygote) and 36.1% (homozygote mutant). PCR amplification was not successful in two samples. Among the 97 patients in the ‘no ART’ control group, 24.8% were wild-type for Asn$^{680}$, 54.6% heterozygote and 20.6% homozygote mutant. No significant differences were found between the ‘OHSS’ and ‘ART/no OHSS’ groups, but there was a more than 40% lower frequency of Ser$^{680}$/Ser$^{680}$ polymorphism in the ‘no ART’ group (Table 1). In contrast, wild-type Asn$^{680}$/Asn$^{680}$ was markedly less frequent in the ‘ART/no OHSS’ and ‘OHSS’ groups (minus 30 and 38% respectively). Performing the Pearson $\chi^2$-test with all three groups together, there was no significant difference.

The distribution of the SNP Cyp19A1 rs10046 was found to be predominantly heterozygous (57.6–63.7%). Of all patients, 19.6–23.5% were homozygous for C/C (wild-type) and 14.3–20.6% homozygous for T/T. The frequency of C/T was higher in patients with OHSS (63.7%) and lower for the homozygous T/T polymorphism (14.3%), although not significant.

Unexpectedly, the frequency of Ile160Thr (heterozygous and homozygous mutated) in patients with ART with and without OHSS was 6.7 and 6.1% respectively. Five patients in the ‘OHSS’ as well as in the ‘ART/no OHSS’ groups were heterozygous and one homozygous for
Thr160/Thr160 (‘OHSS’ group), whereas patients with normal fecundity were all Ile160/Ile160 (wild-type; Table 1).

In the present study, the two patients with spontaneous HL had shown no activating FSHR mutations in exon 10 (Thr449Ile/Ala, Ile545Thr or Asp567Asn/Gly). Heterozygosity could be demonstrated in one patient for Ile160Thr, whereas both presented the homozygous polymorphism Ser680/Ser680 (Table 2).

By combining groups, where applicable, we noted a significant difference between patients with normal fecundity (‘no ART’) and patients treated for sterility (OHSS and ‘ART/no OHSS’ groups combined), with $P = 0.035$ (Table 3). We could show a difference by comparing ART patients and patients with normal fecundity, although not significant. No significant differences were found among IVF patients with or without OHSS.

In a subgroup analysis of the FSHR SNP Asn680Ser, no different distribution of frequencies was found between patients with OHSS grade III and the ‘ART/no OHSS’ group. A trend was observed between patients with OHSS grade II–III and patients with normal fecundity (‘no ART’), being not significant ($P = 0.094$ and 0.099 respectively; Table 3).

Further subgroup analysis revealed significant differences of the FSHR Ile160Thr distribution in the ‘no ART’ group compared with the ‘OHSS’ group, the ‘ART/no OHSS’ group, the ‘OHSS grades II–III’ subgroup or ART group. $P$ values were 0.034, 0.019, 0.026 and 0.039 respectively. No significances or trends were found when comparing the other groups (Table 4).

### Discussion

The SNP frequencies of Ser680Asn in our study were similar to those reported by Daelemans et al. (2004). In a group of 21 women, Greb et al. (2005) analysed the influence of the FSHR genotype in relation to Asn680Ser on menstrual cycle dynamics; the results showed that 12 women (57%) were homozygous for Asn680 and nine (43%) were homozygous for Ser680. Probably due to a less effective receptor protein, the serum levels of oestradiol, progesterone and inhibin A were significantly lower during the luteofollicular transition in women with homozygote mutated Ser680. At the same time, FSH levels were significantly higher and these women had significantly prolonged menstrual cycles. We hypothesize that a lower response due to a conformational change in the FSHR protein might lead to inadequate follicular development and/or follicle recruitment. Perez Mayorga et al. (2000) demonstrated that IVF patients with a homozygous Ser680 polymorphism required significantly more FSH for adequate

<table>
<thead>
<tr>
<th>Table 1 Distribution of genotype frequencies.</th>
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<tr>
<td>SNP</td>
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<tr>
<td></td>
</tr>
<tr>
<td>OHSS</td>
</tr>
<tr>
<td>Asn680Ser</td>
</tr>
<tr>
<td>Ala189Val</td>
</tr>
<tr>
<td>Thr449Ile</td>
</tr>
<tr>
<td>Ile160Thr</td>
</tr>
<tr>
<td>CYP19A1 rs10046</td>
</tr>
<tr>
<td>ART/no OHSS</td>
</tr>
<tr>
<td>Asn680Ser</td>
</tr>
<tr>
<td>Ala189Val</td>
</tr>
<tr>
<td>Thr449Ile</td>
</tr>
<tr>
<td>Ile160Thr</td>
</tr>
<tr>
<td>CYP19A1 rs10046</td>
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<tr>
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</tr>
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<tr>
<td>Ile160Thr</td>
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<td>CYP19A1 rs10046</td>
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ART, assisted reproduction technology; SNP, single nucleotide polymorphism.

<table>
<thead>
<tr>
<th>Table 2 Two patients with spontaneous hyperreactio luteinalis (HL) during early pregnancy.</th>
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<tr>
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<tr>
<td>#4477</td>
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stimulation. Since Thr307Ala has been in linkage disequilibrium with Asn680Ser, it should be possible to compare the frequencies of these two gene variations. In contrast to the findings presented here and also those of Daelemans et al. (2004), d’Alva et al. (2005) did not find different frequencies of Thr307Ala when comparing 50 fertile women (in Brazil) with 29 patients with moderate and severe OHSS.

Although the conditions differ with regard to the time of their occurrence, spontaneous and iatrogenic OHSS share similar pathophysiological sequences, with massive recruitment and growth of ovarian follicles due to non-physiological luteinization. In the iatrogenic form, the pathologic cascade leading to massive fluid shifts is caused by ovarian stimulation with exogenous FSH (Delbaere et al. 2005). In the study by Daelemans et al. (2004), the data show a significantly higher frequency of the FSHR Ser680/Ser680 polymorphism in comparison with the IVF control group, in contrast to the present findings. However, the data presented here confirm a significantly lower Ser680/Ser680 combined with a higher Asn 680/Asn680 frequency in Caucasian control individuals without ART.

Table 3 Comparison of single nucleotide polymorphism (SNP) Asn680Ser frequencies in patients with or without assisted reproduction technology (ART) or ovarian hyperstimulation syndrome (OHSS).

<table>
<thead>
<tr>
<th>SNP</th>
<th>Asn680/Asn680</th>
<th>Asn680/Ser680</th>
<th>Ser680/Ser680</th>
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<tbody>
<tr>
<td>n</td>
<td>%</td>
<td>n</td>
<td>%</td>
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<tr>
<td>OHSS versus no ART</td>
<td>14</td>
<td>15.4</td>
<td>47</td>
</tr>
<tr>
<td>OHSS versus no OHSSa</td>
<td>14</td>
<td>15.4</td>
<td>47</td>
</tr>
<tr>
<td>ART/no OHSS versus no ART</td>
<td>15</td>
<td>17.4</td>
<td>40</td>
</tr>
<tr>
<td>ARTb versus no ART</td>
<td>24</td>
<td>24.7</td>
<td>53</td>
</tr>
<tr>
<td>OHSS grade III versus no ART</td>
<td>10</td>
<td>14.5</td>
<td>36</td>
</tr>
<tr>
<td>OHSS grade II–III versus no ART</td>
<td>24</td>
<td>24.7</td>
<td>53</td>
</tr>
</tbody>
</table>

Values <0.05 considered significant.

aNo ART and ART/no OHSS group. bART: OHSS and ART/no OHSS group.

Table 4 Comparison of single nucleotide polymorphism (SNP) Ile160Thr frequencies in patients with or without assisted reproduction technology (ART) or ovarian hyperstimulation syndrome (OHSS).

<table>
<thead>
<tr>
<th>SNP</th>
<th>Ile160/Ile160</th>
<th>Ile160/Thr160</th>
<th>Thr160/Thr160</th>
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<tr>
<td>n</td>
<td>%</td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>OHSS versus no ART</td>
<td>83</td>
<td>93.3</td>
<td>5</td>
</tr>
<tr>
<td>OHSS versus no OHSSa</td>
<td>83</td>
<td>93.3</td>
<td>5</td>
</tr>
<tr>
<td>ART/no OHSS versus no ART</td>
<td>77</td>
<td>93.9</td>
<td>5</td>
</tr>
<tr>
<td>ARTb versus no ART</td>
<td>160</td>
<td>93.6</td>
<td>10</td>
</tr>
<tr>
<td>OHSS grade III versus no ART</td>
<td>37</td>
<td>97.4</td>
<td>1</td>
</tr>
<tr>
<td>OHSS grade III versus ART/no OHSS</td>
<td>37</td>
<td>97.4</td>
<td>1</td>
</tr>
<tr>
<td>OHSS grades II–III versus no ART</td>
<td>63</td>
<td>94.0</td>
<td>4</td>
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</table>

Values P<0.05 were considered significant.

aNo ART and ART/no OHSS group. bART: OHSS and ART/no OHSS group.

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previously described by Chae et al. (2001). In the present study, the patients with spontaneous HL had no activating FSHR mutations (Thr449Ile/Ala, Ile545Thr or Asp567Asn/Gly). Interestingly, one patient showed a heterozygous Ile160Thr and both had a homozygous Ser<sup>680</sup>/Ser<sup>680</sup>, the latter gene variations with an assumed minimal loss of FSHR function.

Fifty-five cases of HL have been published to date (PubMed search). The aetiology of HL is still unclear but the main aetiological factor for the development of HL could be some intrinsic sensitivity to gonadotrophins causing marked hypertrophy followed by luteinization of the theca-interimna layer, concomitantly with elevated vascular endothelial growth factor (VEGF) levels and other mediators (Haimov-Kochman et al. 2004, Suzuki 2004). The pathological changes that occur in the ovary affected by HL seem to be similar to those seen with OHSS, a condition that typically occurs in the first trimester (Langer & Coleman 2007). Historically, HL was diagnosed typically in the third trimester (54%) during a caesarean section or the puerperium (16%) with bilateral enlargement of the ovaries (Foulk et al. 1997). Only 16% developed HL in the first trimester (Suzuki 2004). Both cases here developed HL around 11th week of gestation with normalization of ovarian size around 21st week of gestation. One may assume different pathophysiological pathways for ‘early’ HL in contrast to ‘late’ HL. Ascites in patients suffering from HL is mostly a rare phenomenon (4%: Foulk et al. 1997). None of the reported cases here presented ascites. HL has been well described as a complication of pregnancy in which there are elevated serum β-hCG levels such as hydatidiform mole and choriocarcinoma (Langer & Coleman 2007). However, HL may rarely occur without elevated β-hCG levels, as described here, too. According to Foulk et al. (1997) and Suzuki (2004), there seems to be an association with maternal hirsutism (14–25%) as was the case with patient #4200. Affected women with gestational hyperandrogenism and HL may have a variety of symptoms including hirsutism (at times requiring shaving), acne, temporal balding, even clitoromegaly and deepening of the voice (Angioni et al. 2007). There are some reports of complications due to rupture of ovarian cysts with subsequent development of an acute abdomen, in agreement with our second case (Morken et al. 2007). No genetic causes are reported to date; although the familial pattern in case 2 would imply such a background.

FSHR Thr449Ile gene variation seems to be a rare mutation described in spontaneous OHSS only once (Vasseur et al. 2003). This gene variation could be detected neither in our patients with iatrogenic OHSS nor in those with HL.

In addition, we could demonstrate the presence of a heterozygous CYP19A1 rs10046 polymorphism in one patient suffering from rather this rare condition. Since CYP19A1 is a polymorphism with a high frequency in the normal population, its implication in this condition remains uncertain.

Greb et al. (2005) investigated the influence of the FSHR Asn680Ser genotype on menstrual cycle dynamics in women. Female menstrual cycle is tightly controlled by pituitary gonadotrophin secretion, which is regulated by ovarian hormones. The secretion of ovarian hormones reflects recruitment of a cohort of antral-stage follicles by elevated FSH concentrations prior to menstrual bleeding. The fate of most of these recruited follicles is atresia. Only one or two follicles selectively become in the end tertiary follicles, with FSH-induced growth and differentiation of granulosa cells. These cells regulate further oocyte maturation (Greb et al. 2005). Even a minute loss of FSHR function due to inactivating gene variants could influence fecundity. Both FSHR polymorphisms investigated here, Ile160Thr and Asn680Ser, may have the potential to do so. Greb et al. (2005) could demonstrate for the first time that patients with the Ser<sup>680</sup>/Ser<sup>680</sup> genotype are more resistant to FSH action and thus require a stronger stimulus for the same biological response. In a follow-up study Behre et al. (2005) were able to show convincingly that patients with the homozygous FSHR Ser<sup>680</sup>/Ser<sup>680</sup> polymorphism required higher FSH dosages in order to show the same oestradiol response during controlled ovarian stimulation. In our study control patients with normal fecundity had a lower genotype frequency of Ser<sup>680</sup>/ Ser<sup>680</sup> in accordance with Daelemans et al. (2004), emphasizing the data of Behre et al. (2005) and Greb et al. (2005). No data concerning the influence of Ile160Thr gene variant on the length of menstrual cycle exists so far.

An altered ovarian reaction may not only be explained by a possible conformational change in the FSHR but also by a subsequently reduced activation of the FSHR-dependent CYP19A1. There is strong evidence that CYP19A1 is involved in the recruitment and selection of ovarian follicles (Bao et al. 1997). As there is a commonly observed association between elevated oestrogen levels and the severity of OHSS, oestriadiol levels could be used as a predictive marker (Rizk 2006). Since a similar genotype distribution of the CYP19A1 rs10046 locus was found in patients with and without ART, there appears to be no association between the CYP19A1 rs10046 marker and iatrogenic OHSS. However, more than 200 other gene variations of the CYP19A1 gene are currently known (de Castro et al. 2005a).

The FSHR gene variation Ala189Val is known to be associated with ovarian dysgenesis in his homozygous form (Aittomaki et al. 1995, Sipila & Aula 2002). However, there was no difference among the groups for the gene variation Ala189Val in our analysis. In contrast, the heterozygous form Ile160Thr is presumed to induce a loss of FSHR function and seems to be more frequent. Beau et al. (1998) found a compound mutation of heterozygous Ile160Thr and Arg573Cys resulting in a
premature ovarian failure (POF). We found no Ile160Thr mutation in the group of patients with normal fecundity, which implies that not only the compound mutations Ile160Thr and Arg573Cys exert an FSHR functional change but also heterozygous Ile160Thr, although far less pronounced. In contrast to spontaneous OHSS, iatrogenic OHSS seems to be multifactorial and not caused by gene variations altering FSHR function. This assumption is sustained by another recent publication in which sequencing of both the FSH and LH receptors of patients with iatrogenic OHSS and controls revealed no differences (Kerkela et al. 2007).

It seems quite tempting to compare clinical data and symptoms compromised by a wide variety of biases in different studies conducted in different countries. In the patients included in the present study, 46.5% (40/86) were classified as having severe OHSS (grade III). All had received hormonal stimulation for IVF, with or without intracytoplasmic sperm injection (ICSI). In contrast to these findings, Abramov et al. (1998a) reported that 7.2% of patients had OHSS grades V and VI. This marked difference can in part be explained by the different classification using six grades commonly referred to in Israel (Rabau et al. 1967).

In agreement with Qasim et al. (1997), Mathur et al. (2000) and Wang et al. (2002), we distinguish between early and late-onset OHSS. Interestingly, Qasim et al. (1997) found that 71.4% of patients with severe OHSS developed late-onset OHSS, in contrast to the findings presented in this study. At the Erlangen University Hospital, 61 of the 86 of patients (70.9%) with OHSS developed early onset OHSS.

With regard to pregnancy rates, Cunha-Filho et al. (2003) reported a pregnancy rate of 52% in women with severe OHSS – slightly higher than the pregnancy rate of 45.3% in the present study, whereas, again, Abramov et al. (1998b) reported a much higher pregnancy rate of 73% in his OHSS patients (grades V and VI).

In conclusion, the FSHR gene and other gene variations may play a role in modulating receptor sensitivity and intracellular second messenger cascades to exogenous hCG and other gonadotrophins. Higher frequencies of wild-type FSHR Asn680Ser and Ile160Thr appear to improve fecundity, but cannot be used to predict the severity of OHSS, given that the number of patients investigated here was rather small. CYP19A1 rs10046 locus seems to have no impact on OHSS or sterility. No polymorphisms could be demonstrated that may lead to the condition of HL.

Materials and Methods

Study design

The study was conducted in collaboration with the Infertility Clinic, Erlangen, Germany. In accordance with Ethics Committee Application no. 3158, 'Molecular Basis for the Aetiology of OHSS', all patients treated in our hospital because of OHSS between 2000 and 2006 received a letter with information, an informed consent and one CDTA blood test tube. If necessary additional phone calls were able to clarify uncertainties. Ninety-one patients with one or more in-patient treatments due to OHSS ('the OHSS group') provided written consent and underwent blood sampling between 2005 and 2006. Clinical data relating to OHSS morbidity, treatment and pregnancies were available for 86 out of the 91 OHSS patients and were obtained either from their respective medical records or by telephone call. The inclusion criteria were status post-OHSS in at least one previous stimulation treatment, age over 18 and no known genomic mutations. All patients, except three Asian women and one African woman, were of Caucasian origin.

Two control groups were established. Eighty-eight control patients (the 'ART/no OHSS' group) who had been treated in the Infertility Clinic, Erlangen, Germany with IVF or ICSI without developing OHSS, were interviewed and consented to undergo an additional blood sample. Ninety-seven parturients admitted to our delivery room (the 'no ART' group), with apparently normal fecundity, who had at least one normal pregnancy and no fertility treatment in their personal history provided written consent to undergo blood sampling. All patients in the control groups except two Asian women in the 'no ART' group were of Caucasian origin. In addition two patients with HL were analysed simultaneously.

Exclusion criteria for the control patients were: status post-OHSS in a previous stimulation treatment, a large number of follicles (>15–20) before aspiration, high oestrogen levels (>4500 pg/ml) before aspiration, known genomic or germline mutations and, for the second control group, any kind of fertility treatment.

To define OHSS, WHO criteria were used as published elsewhere (Delvigne & Rozenberg 2003, Binder et al. 2004). We distinguished between mild (grade I), moderate (grade II) and severe (grade III) OHSS. Statistical analysis was carried out using the Statistical Program for the Social Sciences version 14.0 for Windows. The statistical procedures used for data evaluation were cross-tables combined with the chi²-test (Pearson). For reference purposes, the nomenclature used to describe FSHR gene variations is in accordance with the National Centre for Biotechnology Information (NCBI) protein sequence: P23945.

DNA preparation

Genomic DNA from 8 ml blood was extracted using a genomic DNA purification kit (Puregene, Gentra Systems Inc., Minneapolis, MN, USA), with modifications. Briefly, after initial centrifugation, the white blood cell layer was removed and added to red blood cells (RBC) lysis buffer (pH 7.3) containing 0.15 M NH4Cl, 0.01 M K2CO3 and 0.1 mM Na-EDTA. After 10 min of incubation, the cells were centrifuged at 2000 g and vortexed with a 3 ml cell lysis buffer containing 20 mM Tris, 15 mM Na-EDTA and 1% SDS and treated with RNase A and proteinase K. The proteins were precipitated with 1 ml protein precipitation solution (Puregene) and the DNA precipitated by the addition of isopropanol, washed with 70% ethanol, dried, solubilized
with a Tris-EDTA buffer (pH 7.5) quantitated using a spectrophotometer and stored at −80 °C. Using this methodology, an average of 70–100 μg DNA per patient was obtained.

**Genotyping with real-time PCR**

TaqMan assays for analysis of the FSHR SNP gene variations Asn680Ser (rs6166), Ile160Thr, Ala189Val (in accordance with NCBI protein sequence P23945) and CYP19 rs10046 were purchased from Applied Biosystems Applera Deutschland, Ltd (Darmstadt, Germany). The assay for the FSHR SNP Thr449Ile was designed using the primers: forward, 5′ GGGCAGGCTGTGATGCT and reverse, 5′ GGGTGATAGCTGCTAGAGTGTAGACT; and the allelic-specific primers: 5′ AAAGACAGTGAAGAAG (VIC) and 5′ AAAGACAGTGAAGAAG (FAM). All analyses were performed with the real-time PCR machines ABI7000 and 7300 in accordance with the manufacturer's protocol. Quality control testing of the FSHR Thr449Ile SNP was carried out by Applied Biosystems. Quality control was established in the laboratory using routine tests of DNA from the control group with different SNP assays for homozygosity and heterozygosity. The FSHR gene variations Asp567Asn and Asp567Gly demonstrated non-specific primer binding and thus could not be evaluated with real-time PCR.

**Case reports of two out-patients with HL**

**Case 1**

A 37-year-old German woman (#4477), gravida 1, para 0 was referred to our hospital by her attending obstetrician for further examination at 10 + 6 weeks’ gestation. She had no abdominal symptoms. Vaginal examination revealed a normal cervix, a uterus enlarged to 11 weeks of gestation and non-tender cystic masses palpable in the lower abdomen. Ultrasound examination showed a normal developing foetus, bilaterally enlarged ovaries with multiple cysts (right 5.3 × 4.6 cm). No fluid in the cul-de-sac or signs of a mass were noted. The patient was referred to our hospital by her attending obstetrician and gynaecologist reported scaled-down ovaries with the maximum diameter of 3 cm. Further pregnancy was without complications. The patient delivered a healthy male baby weighing 3150 g and measuring 50 cm. Additionally, the patient reported a familial pattern since her mother experienced the same problems when pregnant with her first child. In 1973 an emergency section had to be performed due to abruption of placenta. Nevertheless, she delivered a healthy girl weighing 3150 g and measuring 50 cm.

In her second pregnancy in 2005, she experienced the same symptoms in early pregnancy. In the 11 + 1 week of gestation, massively enlarged ovaries (14–15 cm) with multiple luteal cysts up to 4 cm were diagnosed. As in the first pregnancy she complained of emesis and discomfort in the lower abdominal region. Only discrete amounts of ascites could be demonstrated. β-hCG was 108.000 mU/ml; TSH, blood count and all other laboratory parameters were within the normal range. Again problems resolved around the 22nd week of gestation. Further, pregnancy was uneventful and in the 37 + 1 week a caesarean section was performed electively. She delivered a healthy girl weighing 2730 g and measuring 46 cm.

In her first pregnancy in 2003, she complained about conspicuously increasing abdominal girth and hyperemesis. Gynaecological and ultrasound examinations in the 11th week of gestation revealed grossly enlarged ovaries with diameters of 12–14 cm and palpable masses in the lower abdomen. Due to the rupture of a cyst (right ovary) and sharp onset of pain she was hospitalized for 5 days. Gradually thereafter, severity of symptoms decreased and after 21st to 22nd week, the size of ovaries was within the normal range. In the 38 + 0 week of gestation an emergency section had to be performed due to abruption of placenta. Nevertheless, she delivered a healthy girl weighing 3150 g and measuring 50 cm.

**DNA sequencing**

In order to find SNPs already demonstrated in patients with spontaneous OHSS or to demonstrate even new gene variants, exon 10 of FSHR (1243 bp, X91747) of patients #4200 and #4477 was amplified from genomic DNA using standard primer and purified. The amplified DNA was then sequenced using different exon 10 primer and ABI Prism BigDye terminator v 1.1 (Applied Biosystems) and analysed on an ABI3100 Avant sequencer (University of Regensburg, Institute for Human Genetics, Germany).

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References


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