Chromosomal and genetic alterations in human hepatocellular adenomas associated with type Ia glycogen storage disease

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Hepatocellular adenoma (HCA) is a frequent long-term complication of glycogen storage disease type I (GSD I) and malignant transformation to hepatocellular carcinoma (HCC) is known to occur in some cases. However, the molecular pathogenesis of tumor development in GSD I is unclear. This study was conducted to systematically investigate chromosomal and genetic alterations in HCA associated with GSD I. Genome-wide SNP analysis and mutation detection of target genes was performed in ten GSD Ia-associated HCA and seven general population HCA cases for comparison. Chromosomal aberrations were detected in 60% of the GSD Ia HCA and 57% of general population HCA. Intriguingly, simultaneous gain of chromosome 6p and loss of 6q were only seen in GSD Ia HCA (three cases) with one additional GSD I patient showing sub-microscopic 6q14.1 deletion. The sizes of GSD Ia adenomas with chromosome 6 aberrations were larger than the sizes of adenomas without the changes ($P = 0.012$). Expression of IGF2R and LATS1 candidate tumor suppressor genes at 6q was reduced in more than 50% of GSD Ia HCA that were examined ($n = 7$). None of the GSD Ia HCA had biallelic mutations in the HNF1A gene. These findings give the first insight into the distinct genomic and genetic characteristics of HCA associated with GSD Ia. These results strongly suggest that chromosome 6 alterations could be an early event in the liver tumorigenesis in GSD I, and may be in general population. These results also suggest an interesting relationship between GSD Ia HCA and steps to HCC transformation.

INTRODUCTION

Hepatocellular adenomas (HCA) are benign tumors that usually develop in women who use oral contraceptives, in patients who receive anabolic/androgenic steroids and in patients with glycogen storage disease type I (GSD I) (1–3). The latter is a rare disease due to a deficiency of glucose 6-phosphatase enzyme activity (GSD Ia), or a deficiency in the microsomal translocase/transport protein for glucose 6-phosphate (GSD Ib) (4,5), resulting in severe hypoglycemia, lactic acidosis, hyperlipidemia and hepatomegaly due to accumulation of fat and glycogen in the liver. With dietary interventions, metabolic control has improved and patients are living into adulthood. With increased survival, long-term complications such as HCA are being increasingly seen (6). In GSD I, HCA typically develop by the second or third decade of life; the risk increases with age and more than 75% patients older than age 30 develop HCA (7). There is no treatment other than resection or liver transplant. The underlying etiology of adenomas in GSD I is unclear, and no significant differences in metabolic control and balance

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has been identified between GSD I patients who develop adenomas and those who do not (8). On the other hand, the presence of adenomas has been found to correlate with serum interleukin 8 concentrations in GSD Ia patients, consistent with the hypothesis that chronic hepatic inflammation that might lead to hepatic injury may predispose these patients to develop HCA (9). GSD I HCA largely shares the clinical presentation, histological features and complications of general population HCA resulting from other etiologies; unlike synthetic sex steroid-induced HCA, GSD I HCA tends to be multiple and show a higher frequency of malignant transformation to hepatocellular carcinoma (HCC) (7,10–13). Distinct chromosomal and genetic alterations could contribute to these differences.

Chromosomal aberrations have been identified in 20–89% of HCAs, obtained mainly from the HCA associated with oral contraceptives (14–16). The average genomic alterations in HCA are much less than those in HCC; and the patterns of genomic alterations between these two pathologic diseases (HCA versus HCC) are also different. Therefore, it seems that only a subset of HCAs with particular characteristics is at high risk of malignant transformation. Interestingly, on the basis of chromosomal aberration patterns detected in HCA with different disease entities, Wilken and colleagues suggested that a progression from HCA to HCC seemed to be possible in the special subset with longstanding and multiple HCAs, but not in the group of HCAs occurring in the typical clinical situation (14,16). Mutation studies revealed that a group of HCAs, representing 15% of cases, exhibiting activating mutations of β-catenin (CTNNB1) were generally characterized by a higher risk of malignant transformation to HCC (17,18). In contrast, biallelic inactivation mutations of HNF1α, a tumor suppressor gene on 12q24.2, although identified in ~35–50% of general population HCA, was less frequently associated with HCC (17–19).

Since there has not been any systematic study published so far, describing chromosomal and genetic alterations in HCA associated with GSD I, due to paucity of sample availability, it is not clear whether the molecular pathogenesis in GSD I HCA is different from that in general population. It is also not clear whether chromosomal alterations in GSD I HCA concur with HCC. Using a sensitive genome-wide high-density SNP analysis and mutation analysis of the two target genes, HNF1A and CTNNB1, we report here the findings of chromosomal and genetic alterations in 10 cases of HCA associated with GSD type Ia. To our knowledge, this is the first detailed, genome-wide study done in a large number of cases with HCA associated with GSD Ia. For comparison, we also studied seven cases of HCA obtained from the general population, who did not have GSD I.

RESULTS

GSD I patient description

The clinical and pathological data from the GSD Ia patients examined in this study are outlined in Table 1. There were six males and four females. Clinical diagnosis of GSD I was confirmed in all patients either through documentation of deficient G6Pase enzyme activity levels on liver biopsy samples (data not shown) or through G6PC gene sequencing of DNA extracted from peripheral blood mononuclear cells. Germline mutation analysis of the G6PC gene revealed that c.247C>T (p.R83C), a common mutation seen in GSD Ia, accounted for 8 out the 20 mutant alleles in our patient group. In contrast, only a mono-allelic missense variant of unknown significance, c.81G>C, resulting in p.Q27H, was detected in one general population adenoma (#18). The average age at adenoma diagnosis was 18 years (range 11–35 years). The final pathology report and diagnosis of nine of these liver tumors was HCA, and for one patient (#37) the final tumor diagnosis was not clearly known. Results of serologic screening tests were available for eight GSD I patients and all of them were negative for HBV and HCV infection.

Chromosomal abnormalities in HCA

By using high density SNP arrays with a median inter-marker distance less than 700 bases, we screened the chromosomal changes for a total of 17 cases of HCA (10 GSD Ia and 7 general population). Among the 17 cases, seven cases (4 GSD Ia and 3 general population) showed adenoma-specific microscopic (≥4 Mb) and submicroscopic (<4 Mb) chromosomal changes, three cases (2 GSD Ia and 1 general population) showed only submicroscopic (<4 Mb) chromosomal changes, yet seven cases (4 GSD Ia and 3 general population) had no detectable chromosomal abnormalities (Fig. 1 and Table 2). When broken down by disease entity, chromosomal aberrations were detected in 60% (6 of 10) of the GSD Ia HCA and 57% (4 of 7) of the general population HCA. However, simultaneous gain of chromosome 6p and loss of 6q were only seen in 3 of the 10 GSD Ia HCA (#s 9, 10 and 37) with one additional patient (#8) showing submicroscopic 6q14.1 deletion (Mb position 79.02–79.08). Alterations of chromosome 6 was the major finding with minimal changes in other chromosomes in three GSD Ia HCA (#s 8, 9, 10), and was significantly associated with larger adenoma lesions (diameter at 10.9 ± 1.8 versus 5.7 ± 2.2 cm, P = 0.012). No other significant correlation between clinical or pathological features and chromosomal aberration pattern was observed (data not shown). Loss of 6q without gain of 6p was observed in one general population case (#18) with multiple chromosomal aberrations, and submicroscopic deletion of two regions on chromosome 6, q13–q14.1 and q24.3–q25.1, were observed in another general population adenoma (#21). Boundaries and genes involved in the genomic regions with gain and loss identified in this study are listed in Supplementary Material, Table S1.

Copy number changes of genomic regions on chromosome 6 were validated using quantitative genomic PCR with primer pairs specific to genes co-localizing to these chromosomal regions, including an angiogenic factor, VEGFA (6p21.1), and three candidate tumor suppressor genes, ZAC1/PLAGL1 (6q24.2), LAT51 (6q25.1) and IGF2R (6q25.3). The results are consistent with copy number estimation by SNP arrays and are shown in Figure 2A–D.

Chromosome 17 abnormality was the second most frequently seen anomaly in HCA (Table 2). 17p11.2–p13.3
including the TP53 locus was deleted in one GSD Ia patient adenoma (#37), one general population adenoma (#20); and it was amplified in another general population adenoma (#18). Additionally common chromosomal aberration involving chromosome 17 was amplification of 17q21.31–q25.3, which was detected in one GSD Ia patient adenoma (#38) and one general population adenoma (#18).

Loss of heterozygosity (LOH) at chromosome 12q has been reported as the only recurrent genetic alteration in a series of HCA samples; however, we could not identify any allelic imbalance of SNPs at any locus on 12q. Moreover, no deletion of the HNF1A gene, a candidate tumor suppressor gene in 12q24, was detected in the 17 cases analyzed here using quantitative genomic PCR (Fig. 2E).

**Expression of candidate genes on chromosome 6 in HCA**

Since deletion of 6q12–q27 were identified in three cases with GSD Ia HCA, we selected two putative tumor suppressor genes co-localizing in this region for further investigation. The IGF2R, localizing at 6q25.3, is a receptor for insulin-like growth factor II (IGF-II) and mannose 6-phosphate. The LATS1 gene, located at 6q25.1, has been suggested to play a role as a tumor suppressor gene through biochemical and genetic evidence. We investigated whether the mRNA levels of these two genes were affected by gene dosage alterations using real-time PCR in liver adenomas and paired normal-liver tissue obtained from seven GSD Ia patients, where good quality RNA samples were available. The results showed that the expression level of IGF2R was reduced in four tumor samples compared with their corresponding non-neoplastic tissues. With the exception of case #36, all the tumors with significantly lower IGF2R expression showed decreased DNA copy number. The correlation between IGF2R expression and DNA copy number was statistically significant (P-value < 0.05) when case #36 was excluded (Fig. 3A). Reduced expression of LATS1 mRNA was also observed in five GSD Ia patients including three with decreased DNA copy number (Fig. 3B). The correlation between LATS1 level and gene dosage of the LATS1 gene was also statistically significant (P-value < 0.05) when the two discordant cases (#s 4, 36) with reduced expression level but without decreased DNA copy number were excluded from the analysis.

**Mutation in the HNF1A and CTNNB genes**

We used PCR amplification and direct sequencing to screen genetic alterations in the HNF1A and CTNNB genes. Biallelic mutations of c.60delG resulting in p.L20fs, and c.344_367dup

Table 1. Clinical, pathological and genetic mutation information of HCA patients associated with GSD-Ia

<table>
<thead>
<tr>
<th>Case #</th>
<th>Gender</th>
<th>Contraceptive use (Age)</th>
<th>Mutations in G6PC</th>
<th>Age (years) at adenoma diagnosis</th>
<th>Adenoma management/age at procedure/years post transplant</th>
<th>Diameter of the main lesion (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>At age 23 years for 5 months</td>
<td>c.1039C&gt;T (p.Q347X)/c.1039C&gt;T (p.Q347X)</td>
<td>20</td>
<td>Transplant; 38 years; 2 years post</td>
<td>4.5</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>None</td>
<td>c.247C&gt;T (p.R83C)/c.247C&gt;T (p.R83C)</td>
<td>13</td>
<td>Liver resection; 16 years; 6 years post</td>
<td>7.3</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>From age 13 years to age 30 years</td>
<td>c.79delC (p.35X)/c.1039C&gt;T (p.Q347X)</td>
<td>15</td>
<td>Liver transplant; 32 years; 3 years post</td>
<td>6.5</td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>N/A</td>
<td>c.563-3c&gt;g/c.1039C&gt;T (p.Q347X)</td>
<td>15</td>
<td>Liver Resection; 45 years; deceased</td>
<td>12.6</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>N/A</td>
<td>c.247C&gt;T (p.R83C)/c.247C&gt;T (p.R83C)</td>
<td>21</td>
<td>Liver resection; 34 years; 2 years post</td>
<td>11</td>
</tr>
<tr>
<td>10</td>
<td>M</td>
<td>N/A</td>
<td>c.979delTT (p.delF327)/c.979delTT (p.delF327)</td>
<td>16</td>
<td>Liver transplant; 31 years; 3 years post</td>
<td>9</td>
</tr>
<tr>
<td>35</td>
<td>M</td>
<td>N/A</td>
<td>c.724C&gt;T (p.Q242X)/c.1039C&gt;T (p.Q347X)</td>
<td>20</td>
<td>Liver resection; 22 years; 2 years post</td>
<td>7.5</td>
</tr>
<tr>
<td>36</td>
<td>F</td>
<td>None</td>
<td>c.247C&gt;T (p.R83C)/c.247C&gt;T (p.R83C)</td>
<td>11</td>
<td>Liver resection; 29 years; 5 years post</td>
<td>6.5</td>
</tr>
<tr>
<td>37</td>
<td>M</td>
<td>N/A</td>
<td>c.247C&gt;T (p.R83C)/c.1039C&gt;T (p.Q347X)</td>
<td>NI</td>
<td>Liver transplant X 3; 35 years; deceased</td>
<td>NI</td>
</tr>
<tr>
<td>38</td>
<td>M</td>
<td>N/A</td>
<td>c.247C&gt;T (p.R83C)/c.562G&gt;C (p.G188R)</td>
<td>Between age 30 and 35 years</td>
<td>NI</td>
<td>1.8</td>
</tr>
</tbody>
</table>

N/A, not applied; NI, no information.

**Figure 1.** Genome view of regions with copy number changes in 17 HCAs. Vertical blue bars represent loss of genetic material in a given tumor, and vertical red bars indicate gains.

> Human Molecular Genetics, 2009, Vol. 18, No. 24
resulting in p.L123_Y130ins of the HNF1A were detected in a general population HCA (#19) (Fig. 4A). A heterozygous silent point mutation (c.870C>T) in the HNF1A gene was detected in adenoma and peripheral blood of GSD Ia case #4, indicating the mutation was germ-line (data not shown). This mutation did not affect splicing sites based on gene structure analysis. No other mutations were detected in the other 15 cases.

Using PCR amplification with primers specific to exon2 and exon4 of the β-catenin (CTNNB1) gene, a smaller fragment of PCR product in addition to the fragment of expected size was detected in GSD Ia case #38, suggesting deletion of exon 3. As expected, HepG2 also showed deletion of exon 3 (Fig. 4B). No point mutations in exon 3 were detected in the other cases using PCR and direct sequencing (data not shown).

**DISCUSSION**

In this report, we present the results obtained through chromosomal aberration study done on 10 cases with GSD type I HCA and seven cases of general population HCA, using a high-density SNP array. This methodology is a very sensitive way to detect submicroscopic changes in a chromosomal segment, as small as 35 kb. We identified chromosomal aberrations in 60% of the HCA from GSD Ia and 57% of HCA obtained from the general population. This frequency is similar to what has been reported previously for general population HCA (14–16). Among the aberrant chromosomal regions identified, four were <100 kb in size (Supplementary Material, Table S1), indicating that using the high-density SNP array, chromosomal aberration involving just a single gene can be identified.

Chromosomal and genetic alterations in HCA reported by other groups and in this study are summarized in Table 3 (14–17,20). A major difference from previous studies, in which gain of 17q and 20q were the most commonly observed chromosomal alterations in HCA obtained from general population, we have observed that in our series of GSD Ia adenomas the most significant chromosomal aberration is on chromosome 6, with simultaneous gain of 6p and loss of 6q. Although loss of 6q without gain of 6p was identified in two general population HCA in this study, gain of 17q and 20q were the most commonly observed chromosome 6 alterations in HCA obtained from general population. We have observed that loss of 6q and/or simultaneous gain of 6p could be an early event in the liver tumorigenesis in GSD I, and may be in general population. Moreover, the simultaneous gain of 6p and loss of 6q has been reported as two of the most frequent events in HCC. Therefore, we speculate that GSD I HCA with simultaneous gain of 6p and loss of 6q could be at high risk for malignant transformation, and genes in chromosome 6 could be implicated in the sequel of HCA to HCC transformation. Although none of the nine cases with known pathological diagnosis showed evidence of malignancy, 'nodule-in-nodule' lesions is still a possibility due to the number of adenomas these patients have and considering the fact that only a limited

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**Table 2. High-density SNP array results on the chromosomal aberrations in hepatocellular adenomas**

<table>
<thead>
<tr>
<th>Disease</th>
<th>Case #</th>
<th>Chromosomal gain</th>
<th>Chromosomal loss</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSD-Ia</td>
<td>1</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>6p24.3–q12</td>
<td>6q12–q27</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>1p31.3*</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>4q32.1*, 5p15.33–q23.3, 6p25.3–q11.1, 6q12*, 6q13*</td>
<td>6q12–q27</td>
</tr>
<tr>
<td>General</td>
<td>38</td>
<td>17q21.31–25.3</td>
<td>None</td>
</tr>
<tr>
<td>population</td>
<td>16</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>17p/q</td>
<td>6q12–27</td>
</tr>
<tr>
<td></td>
<td>19</td>
<td>None</td>
<td>10q22.3, 12p11.21–p11.1*</td>
</tr>
<tr>
<td></td>
<td>44</td>
<td>None</td>
<td>None</td>
</tr>
</tbody>
</table>

*Involved region is smaller than 4 Mb.

Regions with bold face are detected in more than one case.
number of sections undergo histological analysis. Intriguingly, although the pathological diagnosis of case #37 was not clearly known, the existence of gain of 6p, loss of 6q, and multiple other chromosomal abnormalities raises the possibility that this case could have undergone chromosomal instability, and possibly HCC transformation.
LOH of several putative tumor suppressor genes on 6q, including IGFR2 on 6q25.3 and LATS1 on 6q25.1, have been reported to be associated with HCC and other cancers (21–23). Furthermore, frequent LOH of IGFR2 has been reported in dysplastic liver lesions as well as in HCCs, and the protein expression has been found to be largely reduced in HCC cells (24,25). These results not only strongly suggest that IGFR2 is a tumor suppressor gene but also provide evidence that its inactivation/haploid insufficiency is an early event in liver carcinogenesis. In our study, reduced IGFR2 mRNA level was identified in four out of the seven HCA associated with GSD Ia, strongly suggesting that inactivation of IGFR2 is most likely responsible for tumorigenesis in this type of HCA and these HCA are at increased risk for malignant transformation. The major molecular mechanism for reduced expression of IGFR2 in the GSD Ia HCA in this case series is decreased gene dosage (#s 9, 10 and 37); whereas post-transcriptional regulation may be responsible for that in case #36 because amplification of has-mir-101–1, a microRNA which was predicted to bind to the 3'-UTR of IGFR2 and suppress its expression was identified in this case (Supplementary Material, Table S1). One function of IGFR2 is binding to and sequestering IGFR-II for protein degradation (26), and loss of IGFR2 expression results in increased serum and tissue levels of IGFR-II in mice (27). Interestingly, increased extracellular supply of IGFR-II would lead to tumor progression (28,29). Therefore, for the cases of HCA associated with increased IGFR-II availability caused by inactivation of IGFR2, IGFR-II-targeted therapy is possibly an alternative treatment to prevent malignant transformation.

The LATS1 gene, one of the core components of the Hippo pathway, has been identified as a tumor suppressor in Drosophila and mice (23,30). LATS1 inactivates oncogenic function of YAP, the nuclear effector of the Hippo pathway. Constitutive over-expression of YAP in transgenic mice enhanced proliferation of hepatocytes and eventually induced nodules of HCC (31). Thus, loss of function of LATS1 could contribute to overgrowth of liver cells. Reduced LATS1 mRNA expression in five out of the seven GSD Ia HCA strongly suggests that LATS1 is involved in the development of this subtype of HCAs. Two GSD I cases (#4 and #36) with decreased LATS1 expression did not show any DNA copy number change, strongly suggesting that mechanisms other than deletion are responsible for down-regulation of LATS1. Epigenetic modification is a possible mechanism since promoter hypermethylation has been reported to mediate down-regulation of LATS1 mRNA expression in human breast cancer (32).

HCCs with gain of 6p are associated with large size of tumor and local invasion (33,34). Therefore, candidate oncogenes on 6p can be putative therapeutic targets for HCC. Interestingly, significant over-expression of the VEGF gene on 6p21 was only reported in HCCs with high-level gains at 6p21, but not in tumors with gain of chromosome 6 (35). Consistent with the findings by Chiang et al., we did not observe an association between the VEGFA gene expression and gain of 6p in this study. Thus based on our findings and those previously reported, VEGFA does not appear to be a good biomarker or therapeutic target for GSD I HCA.

In summary, isolated microscopic and submicroscopic chromosomal aberrations involving simultaneous gain of chromosome 6p and loss of 6q are the most common genetic aberrations seen in HCAs associated with GSD Ia, and underscore the significance of chromosome 6 aberrations in liver tumorigenesis in GSD Ia. It is likely that cooperative activation of multiple oncogenes on 6p and inactivation of multiple tumor suppressor genes on 6q contribute to the development of HCAs in GSD Ia patients. Moreover, since gain of 6p and loss of 6q is frequently identified in HCC, it will be important to investigate the involvement of genes on this chromosome in malignant transformation of HCA. Although our findings could not distinguish adenomas that will stay benign from those that will undergo malignant transformation, the high frequency of GSD Ia HCA with chromosomal and genetic aberrations that are associated with HCC in the general population justifies removal of the adenomas to prevent possible development of HCC, if left unattended. Our experience of HCA resection in GSD Ia patients demonstrates that partial hepatectomy is feasible in these patients and is an effective intermediate step in the prevention of HCC until definitive treatment such as a liver transplant (36). Patients with these high-risk aberrations may be good candidates for liver transplant until we have a better understanding of the pathogenesis and other therapeutic targets (37).
A study with larger number of cases and detailed histopathological analysis is needed for better understanding of HCA–HCC progression and treatment management in GSD I. Nevertheless, the findings in this report give the first insight into the distinct genomic and genetic characteristics of HCA associated with GSD Ia.

MATERIALS AND METHODS

Patient tissue samples

Paired samples of HCA and adjacent normal liver tissue sample, and peripheral blood samples were collected from 10 patients with GSD Ia undergoing hepatectomy and/or liver transplant between years 2000 and 2008, at Duke University Medical Center and other collaborating medical centers. Additionally seven de-identified HCA tissue samples were obtained from the sample bank at Duke University Medical Center from patients undergoing routine resection or hepatectomy for adenomas unrelated to GSD I. These samples were collectively labeled as general population HCA. The specimens were frozen immediately after resection in liquid nitrogen and stored at $-80^\circ$C until used. All samples were obtained following informed patient consent and under Institutional review board approved protocols obtained at each institution.

DNA preparation

Genomic DNA was isolated from peripheral blood, liver adenomas and the corresponding non-neoplastic tissues, using Puregene genomic DNA purification kit (Gentra Systems,
Chromosomal and genetic alterations identified in GSD I-associated and general population HCA

<table>
<thead>
<tr>
<th>Reference</th>
<th>This study</th>
<th>Zucman-Rossi et al. (17)</th>
<th>Chen et al. (20)</th>
<th>Chen et al. (15)</th>
<th>Winkens et al. (14)</th>
<th>Steinemann et al. (16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methods</td>
<td>SNP array, genomic sequencing, PCR amplification</td>
<td>Sequencing, PCR amplification</td>
<td>Sequencing, PCR amplification</td>
<td>CGH</td>
<td>CGH</td>
<td>Array CGH</td>
</tr>
<tr>
<td>Samples</td>
<td>GSD-I (n = 10)</td>
<td>General population (n = 7)</td>
<td>GSD-I (n = 2)</td>
<td>General population (n = 94)</td>
<td>General population (n = 10)</td>
<td>General population (n = 10)</td>
</tr>
<tr>
<td>Bi-allelic inactivation of HNF1A</td>
<td>0</td>
<td>14% (1)</td>
<td>0</td>
<td>0</td>
<td>47% (44)</td>
<td>ND</td>
</tr>
<tr>
<td>β-catenin mutated</td>
<td>10% (1)</td>
<td>0</td>
<td>50% (1)</td>
<td>13% (12)</td>
<td>30% (3)</td>
<td>ND</td>
</tr>
<tr>
<td>Chromosomal aberration</td>
<td>60% (6)</td>
<td>57% (4)</td>
<td>ND</td>
<td>ND</td>
<td>75% (6)</td>
<td>20% (2)</td>
</tr>
<tr>
<td>Chromosomal aberration (average event)</td>
<td>3.2</td>
<td>2.7</td>
<td>ND</td>
<td>ND</td>
<td>4.7</td>
<td>0.4</td>
</tr>
<tr>
<td>Gain of 6p and loss of 6q</td>
<td>30% (3)</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
<td>25% (2)*</td>
<td>0</td>
</tr>
<tr>
<td>Gain of 17q</td>
<td>10% (1)</td>
<td>14% (1)</td>
<td>ND</td>
<td>ND</td>
<td>50% (4)</td>
<td>10% (1)</td>
</tr>
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</table>

( ): Number of cases.
* The cases showed multiple chromosomal alterations.
ND: Not done.

High-density oligonucleotide array and data analysis

A total of 500 ng of genomic DNA of each sample was subjected to SNP genotyping using Genome-wide Human Array SNP6.0 (Affymetrix, CA, USA) according to the manufacturer’s instructions. Genotyping was performed by the National Genotyping Center at Academia Sinica, Taipei, Taiwan (http://ngc.sinica.edu.tw). Copy number estimation, LOH analysis, and allele difference analysis for both GSD Ia HCA and paired normal tissue and general population HCA was carried out with Genotyping Console Version 2.0 (Affymetrix, CA, USA) under unpaired mode by using a reference set of 232 healthy individuals genotyped at the same center. Copy number estimation, allele ratio analysis, and alleled-specific copy number analysis for GSD Ia HCA was performed using Partek Genome Suite (Partek Inc., MO, USA) under paired mode by comparing genotyping data from adenomas to that from corresponding normal blood/liver tissues to remove germ-line copy number variations (CNV). For the general population cases, because of the unavailability of paired normal tissues, data analysis using Partek Genome Suite was performed under unpaired mode. Aberrant regions identified in general population HCA showing more than 50% overlapping with CNVs reported in Database of Genomic Variants (http://projects.tcag.ca/variation/) were removed from the final report to eliminate putative germ-line CNV. Regions where at least 50 consecutive probes with the same direction of copy number change identified by both analytic algorithms, were defined as having amplification or deletion changes. The selected regions were further manually corrected according to the results of allele ratio and allele specific copy number to link two isolated regions that were separated by <1 Mb into one continuous region.

Quantitative genomic PCR

Primer pairs specific to genomic sequences of the VEGFA, ZAC1/PLAGL1, LATS1, IGFR2, and HNF1A genes were designed using Primer 3, and the primer sequences are described in Supplementary Material, Table S2. Quantitative genomic PCR was performed following the procedure as described previously (38), except that GAPDH gene instead of the LINE1 gene was used for internal control in this study. Thermal cycling condition was also modified as the follows: initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and combined annealing and extension at 60°C for 1 min. All reactions were performed at least two times and the mean values were reported.

Quantitative RT–PCR

Total RNA was isolated from tumor and non-neoplastic tissues using TRI Reagent (Ambion, Austin, USA). Two microgram of total RNA was used for reverse transcription using Transcriptor Reverse Transcriptase system (Roche Applied Science, Indianapolis, USA). Primer pairs specific to cDNA sequences of the VEGFA (NM_001025367), LATS1 (NM_014572) and IGFR2 (NM_000876), were designed using Primer 3, and the primer sequences are described in Supplementary Material, Table S3. A final volume of 10 µl per reaction contains 60 ng of cDNA, 1 x SYBR Green master mix (Applied Biosystems, CA, USA), and 250 nM of primers. The reaction was carried out as described for quantitative genomic PCR. All reactions were performed at triplicate for at least two times and the mean values were reported. The relative expression level of target genes in each sample was determined by calculating ΔCt values between target genes and GAPDH. Then ΔΔCt values were calculated between the ΔCt for a tumor sample and the ΔCt for the corresponding non-neoplastic tissue.
Statistical analysis

Fisher’s exact test was used to detect difference of dichotomous characteristics between groups (gender and mutations identified for c.247C>T and c.1039C>T), whereas Wilcoxon two-sample test was perform for continuous variables (age at adenoma diagnosis and diameter of the main lesion). A difference showing a two-side P-value < 0.05 was set to be significant. The analyses were carried out using SAS version 9.1 (SAS Institute, Cary, NC, USA). Linear regression models were fitted to access the effects of the quantified gene dosage on mRNA expression level for IGF2R, LATS1 and VEGFA. The relation was defined as significant when P-value < 0.05.

Mutation analysis

Primer sequences for amplification and sequencing of exons for the G6PC, HNF1A and β-catenin genes, and reaction condition for PCR are described in Supplementary Material, Table S4. Direct sequencing of PCR products was performed using ABI sequencer 3730 (Applied Biosystems, CA, USA). Data analysis was carried out with Sequencher, version 4.2 (Gene Codes Corporation, Ann Arbor, USA). All mutations were determined by sequencing PCR product from two independent PCR amplifications. Interstitial deletion analysis of the β-catenin gene was done by PCR amplification of exons 2 through 4 with primers: 5′ ATGAGGCTGCGTTTCACTAAACC and 5′ GGTATGGGTTAGACATTTGCAAAC TACT. The PCR products were electrophoresed in a 1.5% agarose gel.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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