Genome-Wide Analysis of DNA Copy Number Changes in Liver Steatosis

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Authors’ contributions

This work was carried out in collaboration between all authors. Authors FR and LAP designed the study; authors FR, AZ and NP performed the analysis; authors FR, FA, JCC, JLZ and LAP did the statistic analysis; authors JJE and IZ performed the histological and immunohistochemical studies, author FR wrote the first draft, author LAP wrote the manuscript. All authors read and approved the final manuscript.

ABSTRACT

Aims: Liver steatosis is the most common benign form of non-alcoholic fatty liver disease. It might be a risk factor for hepatocellular carcinoma, either (i) by causing fibrosis, which highly predisposes to hepatoma, or (ii) by being an early precursor of carcinoma, although it is usually considered not to be pre-neoplastic. We investigated...

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the genomic profile of liver samples from patients with fatty liver disease.

**Study Design & Methodology:** Copy number variation was investigated by array-CGH, using the Human Genome 244K catalogue array (Agilent Technologies), and changes validated by quantitative polymerase chain reaction analysis.

**Results:** The analysis of liver biopsies from 17 patients, 10 of whom had histological diagnosis of non-alcoholic fatty liver disease, showed differences in the type of variants in patients with steatosis compared to those without steatosis at several chromosome bands, including 3q29, 6p2, 11q11 and 22q11.

**Conclusion:** The genomic copy number changes we have demonstrated suggest that genomic structural variations may be associated with the pathogenesis or the evolution of steatosis.

**Keywords:** Array-CGH; chromosome; CNV; genomic profile; liver steatosis.

1. **INTRODUCTION**

Liver steatosis is the most common benign form of non-alcoholic fatty liver disease (NAFLD). Accumulation of triglyceride within hepatocytes (i.e., steatosis) is the hallmark of NAFLD. Non-alcoholic steatohepatitis (NASH) is a more advanced lesion of intermediate severity, in which steatosis is accompanied by liver inflammation, hepatocyte injury and death [1]. Hepatocellular carcinoma (HCC) lies on the opposite end of the spectrum where most liver-related morbidity and mortality occur. HCC development has been described as a multistep process, starting from pre-neoplastic lesions, among which dysplastic nodules (DNs) are the most frequent lesions associated with HCC [2]. Studies by conventional and molecular cytogenetics have revealed that, as in most tumour types, genetic abnormalities play a pivotal role in the development of HCC [3,4]. Furthermore, genome-wide screening by comparative genomic hybridisation (CGH) of benign liver lesions, such as focal nodular hyperplasia (FNHs), hepatocellular adenoma (HCAs), and DNs have shown that FNHs and HCAs exhibited few chromosomal abnormalities, whereas the genomic profile of DNs highly resembled those in HCCs [5].

NAFLD is the most common cause of chronic liver disease in many western countries, and frequently NASH-related liver damage triggers liver fibrosis, which highly predisposes to HCC [6]. Furthermore, hepatic lipid peroxidation has been shown to increase the intracellular level of reactive oxygen species, leading to oxidative stress, which is theorized to promote carcinogenesis [7].

In the present study, we investigated the genomic profile of fatty liver disease by array-CGH, using samples from patients subjected to liver biopsy to confirm fatty liver disease. Such biopsies are routinely available from NAFLD patients, because in spite of the improvements in non invasive diagnostic techniques, liver biopsy remains the preferred procedure for the establishment of diagnosis in cases of abnormal liver function tests, cholestatic diseases, or the suspicion of neoplastic diseases, and NAFLD [8]. Array-CGH showed differences in copy number at several loci between patients with and without steatosis.
2. METHODOLOGY

2.1 Patients

The study included 17 samples from patients who underwent liver biopsy at the Department of Surgery of the Galdakao Hospital, Basque Country, to confirm liver steatosis. They were free of liver tumour at the time of the examination.

The approval for the study was provided by the Ethical Committee of the Basque Country Department of Health, and written informed consent was obtained from all patients, according to the Declaration of Helsinki Principles.

2.2 Histological and Immunohistochemical Studies

Each specimen was divided into two parts: one for histopathologic examination by standard hematoxylin-eosin staining, and one for a-CGH analysis, which was immediately snap frozen and conserved until the analysis were performed.

2.3 Array-CGH Analysis

Liver tissue samples were homogenised with a pestle and DNA extraction was performed using the Blood & Tissue kit (Quiagen, USA), according to the manufacturer’s specifications. We purposefully selected commercial DNA (Promega, USA) from healthy males or females as controls to be able to detect CNVs. Approximately 500 ng of DNA from each sample, and gender-matched reference DNA, were digested with Rsa I and Alu I restrictions enzymes. Extent of the digest and the quality of the DNA were checked by capillary electrophoresis in a Bioanalizer 2100 (Agilent Technologies, France). Then, sample and reference DNA were labelled, purified, and hybridized on a Human Genome 244K catalogue array (Agilent Technologies, France) according to the instructions described in www.agilent.com. The data was extracted using the Feature Extraction v.9.5.1 software and analysed using DNA Analytics v.4 software (ADM-2) from Agilent Technologies. Only hybridizations with a Derivative Log Ratio Spread (DLRS) value < 0.3 were used for data collection. A minimum log ratio of 0.25 in at least 3 consecutive probes was established as criterion to consider a deviation as an aberration. Moreover, a maximum of 100 aberration regions per genome was allowed. Fuzzy zero and centralization algorithms were applied to avoid extended aberrant segments with low absolute mean ratios that usually represent noise, and are detected because of high number of probes in the region, according to the manufacturers. Complete a-CGH data are available upon request.

2.4 Quantitative RT-PCR for Expression Analysis

RNA was isolated from samples Bio106, Bio113, Bio119, and from the hepatoma cell line HepG2, using the RNAeasy Mini kit (Qiagen, USA) and treated with RNAase-free DNAase set (Qiagen, USA). The HepG2 cell line was purchased from American Type Culture Collection (ATCC, USA) and maintained in D-MEM High Glucose medium (GIBCO, Germany) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin at 37 °C in a humidified 5% CO₂ atmosphere. The cDNA was synthesized from 1 μg of RNA using the Reverse Transcriptase kit (Promega, USA) following the manufacturer recommendations. qRT-PCR was performed using the SYBR Green PCR Master Mix (ABgene, UK) in an iCycler thermocycler (Bio-Rad Laboratories Inc, UK) following the instructions of the
manufacturer. Primers sequences and annealing temperatures for SLC10A1, RPLP0 genes were described elsewhere [9,10]. The sequences for SLC10A1 primers were; F: GGACATGAACCTCAGCATG and R: AATGAGAACCAGGACCAGTG. The sequences for RPLP0 were F: CGACCTGGAAGTCCAACTAC and R: ATCTGCTGCATCTGCTTG. The efficiency of each set of primers was determined by serial dilution of the template cDNA. The theoretical value obtained with the iCycler software was 101% and 103% respectively (Efficiency (%) = 100x [10^{1/slope} – 1]; Bio-Rad Laboratories Inc). Gene expression level was estimated as the difference of the Threshold Cycle value (C_T) obtained for the SLC10A1 gene in the sample and the C_T values yielded by the biopsy sample with the lowest transcriptional level (BIO106), normalized respect the expression level of the housekeeping gene RPLP0. The data was calculated according to the formula 2^{ΔΔCt}, in which ΔΔCt= (CtRPLP0 Bio106 – CtRPLP0 sample) – (CtSLC10A1 Bio106 – CtSLC10A1 sample).

2.5 RT-PCR for Copy Number Validation of GSTT1 Gene

We selected the glutathione S-transferase theta 1 (GSTT1) for validation because variations in the copy number of detoxification genes have been related to liver pathology, included cancer development or progression. Validation was performed using genomic DNA from liver tissue and blood of all patients, except one whose blood sample was not available. The quantitative polymerase chain reaction analysis was performed using the Taqman Copy Number Assay (Applied Biosystems, UK). DNA samples were diluted to a concentration of 5 ng/ul, and a total 20 ng was used for each reaction, performed as multiplex assay with AB reagents and using an iCycler machine (Bio-Rad Laboratories Inc, UK). The control gene, RNaseP (Taqman Copy Number Reference Assay, UK), was labeled with VIC fluorochrome and the GSTT1 amplicons were labeled with FAM fluorochrome. The efficiency of the commercial probes in our system was confirmed to be nearly 100%. Relative quantification was performed by the ΔCt method (ΔCt= Ct gene – Ct RNaseP), and the number of copies (CN) was calculated as 2x2^{ΔCt}. CN values between 1.2 and 2.5 were considered indicative of 2 copies, and correspond to no-change by aCGH analysis. Whereas CN values between 2.5 and 3.5 correspond to 3 copies, and it is associated with a positive value by aCGH. Absence of amplification by Taqman technology corresponds to negative results by aCGH (lower than -3), and they can be interpreted as the lack of the gene in the sample.

3. RESULTS

3.1 Clinical and Histological Findings

The study included 17 patients, nine female and eight male, mean age 58 years (range 30-85 years), all negative for HBV (HBsAg) and HCV (anti-HCV negative). By histology, 10 had steatosis (Fig. 1). After 2 years follow-up, two patients died of pancreatic cancer. Pertinent clinical and histological features are presented in Table 1.
3.2 Array CGH Results

All samples showed an aberrant profile by aCGH analysis, the copy number changes ranged from 3 to 35 per sample (Fig. 2A), and affected all chromosomes. However, some genome regions were particularly involved in variations, including 1p36, 1q21, 3q26, 3q29, 4q13, 4q34, 6p21, 8p11, 9q22, 11q11, 12p13, 14q11, 14q32, 17q21, 22q11, and 22q13, which were observed in ≥ 5 patients (Fig. 2B). To further confirm this, we reanalyzed the data using a more restrictive criterion to accept a deviation as an aberration, a change in log ratio of 0.5, and we found differences in the type of variants in patients with steatosis compared to those without steatosis. Lower copy number at 3q29 was found almost exclusively in patients with steatosis (6/10). Chromosome bands 6p21 and 11q11 exhibited copy number losses in 50% of the patients with steatosis, whereas gains were detected in 42% of the samples from patients without steatosis. On the contrary, at the genome region 22q11 the profile of genome variation was the opposite, gains were detected in five out of 10 patients with fatty liver disease and losses in two out of seven patients without cellular triglyceride accumulation (Fig. 2B). These include genomic regions in which copy number polymorphisms (CNV) have been described (Database of Genomic Variants, http://projects.tcag.ca/variation/). Therefore, such variations do not necessarily represent genome aberrations related to the disease. However, it is noteworthy that several of the detected CNVs overlapped or encompassed genes found to be associated with liver disease or cancer development. For example, MUC20, a negative regulator of the HGF-induced Grb2-Ras pathway maps at 3q29, and Glutathione S-Transferase Theta 1 (GSTT1) mapping to 22q11 (Fig. 2C).
Table 1. Age and sex of the patients, factors associated with the development of disease.

| Patient | Gender/Age | Clinical Parameters | Steatosis | Inflammation | Fibrosis | Cholesterol | Triglycerides | Glucose | Cancer history | Others |
|---------|------------|---------------------|-----------|--------------|----------|-------------|---------------|---------|---------------|--------|
| Bio103  | F/82       |                     | +         | -            | -        | H           | H             | N       | DMNoID        |        |
| Bio106  | F/65       |                     | +         | -            | -        | H           | N             | N       | DMNoID        | Pancreas cancer |
| Bio107  | F/72       |                     | +         | -            | -        | H           | N             | N       | DMID          | Pancreas cancer |
| Bio108  | M/52       | ++                   |           | -            | -        | N           | N             | N       | N             | Bladder cancer |
| Bio112  | F/69       | +                    |           | -            | -        | N           | DMNoID        | N       | Cancer history | Others |
| Bio113  | M/39       | ++                   |           | -            | -        | N           | N             | N       | Chronic hepatitis (Gr 1, St 2) |
| Bio120  | F/53       | +                    |           | +            | ++       | H           | N             | N       | Chronic hepatitis (Gr 1, St 1) |
| Bio121  | M/30       | +                    |           | -            | N        | N           | DMoID         | N       | Ampuloma       |        |
| Bio122  | M/39       | +++                  |           | +            | +        | N           | N             | N       | Primary bile duct cirrhosis |        |
| Bio123  | M/46       | ++                   |           | +            | -        | H           | N             | N       | Biliary lithiasis |        |

(-) Negative, (+) Mild, (+++) Severe, (H) High, (N) Normal, DMNoID: Diabetes mellitus no insulin dependent, DMID: Diabetes mellitus insulin dependent
Fig. 2. A) Frequencies of copy number changes in patients with steatosis (purple) and patients without steatosis (blue). Columns give the total number of variations observed in each sample. B) Overview of chromosome regions variations detected in ≥ five samples. Patient number and chromosome sites are indicated horizontally and vertically, respectively. Log ratio of 0.5 in at least 3 consecutive probes was established as criterion to consider a deviation as an aberration, a threshold value higher than the commonly used in a-CGH studies of cancer cells. Chromosome alteration groups: green, gain; red, losses, yellow, indicates that at this band there have been gains of some probes and losses with others. C) Detail of chromosome bands and genes with contrasting profile of copy number variations between patients with and without steatosis.

The analysis also revealed that several individuals had genome aberrations not listed as CNVs. Two patients without steatosis (Bio119 and Bio123) had gains of genetic material at 14q24.2, the locus of the DCAF4 gene, while one patient with liver accumulation of triglycerides had losses at 5q31.3 (GNPDA1) (Table 2). One patient, who suffered of obstructive biliary lithiasis (Bio119), had several genomic imbalances, including gains at 2q11.2, 14q24.2 and 19q24.2, and losses of genetic material at 14q24.1, the locus of the SLC10A1 gene (Table 2). We therefore asked whether this structural change has any effect on gene expression and we determined the transcription level of this gene by qRT-PCR. Intriguingly, the analysis revealed that the expression level of SLC10A1 gene in the liver sample was higher than the transcription level in two patients and a HCC cell line (HepG2) with normal SLC10A1 gene dosage (Fig. 3).
Table 2. Genome aberrations in individual patients

| Patient | Steatosis | Chromosome band | Log ratio | Gene                                                                 |
|---------|-----------|-----------------|-----------|----------------------------------------------------------------------|
| Bio113  | ++        | 5q31.3          | -1.0      | Glucosamine-6-Phosphate Deaminase 1 (GNPDA1)                         |
|         |           | 2q11.2          | 1.0       | Protein LOC150763 (LOC150763)                                        |
|         |           | 14q24.1         | -1.0      | Sodium/Bile Acid Cotransporter Family, Member 1 (SLC10A1)            |
| Bio119  | -         | 14q24.2         | 1.2       | WD repeat-containing protein (DCAF4)                                 |
|         |           | 19q24.2         | 1.0       | COMM Domain Containing 4 (COMMD4)                                    |
| Bio123  | -         | 14q24.2         | 1.3       | WD repeat-containing protein (DCAF4)                                 |
|         |           | 2q11.2          | -1.2      | Protein LOC150763 (LOC150763)                                        |

Fig. 3. qRT-PCR for SLC10A1, using as templates the cDNA synthesized from RNA extracted from samples Bio106, Bio113, Bio119, and the cell line HepG2. mRNA levels were quantified, normalized against the mRNA level of the RPLP0 gene, and are expressed as fold changes respect to Bio106.

3.3 GSTT1 Copy Number Alteration

The GSTT1 gene mapping to 22q11 was selected as candidate gene for validation because this region was recurrently altered in patients with steatosis. Validation was performed using genomic DNA from liver tissue and blood samples. Except for two patients (Bio105 and Bio106) we obtained good correlation, as to the copy number of GSTT1, between the liver and the blood samples using this technology (Table 3). Taqman analysis revealed copy gains of the gene in the liver sample of Bio105, which was not detected in blood, and the opposite was obtained for the samples from Bio106. GSTT1 copy number gains, detected by aCGH, were confirmed in four out of five liver samples from patients with steatosis (Bio103, Bio108, Bio121 and Bio124), and also the loss of material from this genomic region.
in the two patients without steatosis. Patient Bio 113 exhibited a gain of \textit{GSTT1} by aCGH, however the Taqman assay did not show increase in number of copies, probably reflecting differences in the sensitivity between the two types of methods. In fact, and contrary to Taqman assay, aCGH includes several probes covering this gene.

### Table 3. Data from Taqman assay for the \textit{GSTT1} gene

| Patient | Steatosis | Taqman assay | aCGH assay |
|---------|-----------|--------------|------------|
|         |           | Liver | Blood | Liver |
| Bio103-2| +         | 3.05  | NS    | 0.83** |
| Bio106-5| +         | 1.75  | 3.53§ | 0**  |
| Bio107-6| +         | 2.86  | 3.42  | 0*   |
| Bio108-7| ++        | 3.12  | 3.20  | 0.77**|
| Bio112-9| +         | 1.48  | 1.90  | 0**  |
| Bio113-10| ++       | 1.42  | 1.85  | 0.61* |
| Bio120-13| +        | 2.71  | 5.12  | *    |
| Bio121-14| +        | 2.81  | 3.30  | 0.65* |
| Bio122-15| +++      | 1.44  | 1.48  | 0**  |
| Bio124-17| ++       | 3.05  | 2.64  | 0.90**|
| Bio102-1 | -         | 1.61  | 1.91  | 0**  |
| Bio104-3 | -         | NA    | NA    | -3.68**|
| Bio105-4 | -         | 2.86  | 1.74§ | 0.53**|
| Bio109-8 | -         | 1.96  | 1.52  | 0**  |
| Bio117-11| -         | NA    | NA    | -3.61**|
| Bio119-12| -         | 1.21  | 2.30  | 0**  |
| Bio123-16| -         | 1.90  | 2.05  | 0**  |

Values of the Taqman assay ranging 1.2 - 2.5 correspond to 2 copies, and values ranging 2.5 - 3.5 indicate the presence of 3 copies. A positive value obtained with aCGH analysis indicates 3 copies, whereas a value < -3 the absence of the gene.

ND: No sample
NA: No amplicons
§: Disagreement between liver and blood with Taqman assay
*: Disagreement between Taqman assay and aCGH for liver samples
**: Agreement between Taqman assay and aCGH for liver samples

### 4. DISCUSSION

Comparative genome hybridization allows scanning of the entire genome for unbalanced chromosome aberrations and normal CNVs [11]. Normal CNVs are segments of DNA ranging from 1 kilobase (kb) to several megabases (Mb), for which copy-number differences have been found among phenotypically normal individuals [12,13]. Since these initial reports, a large number of studies have described the wide spread and global distribution of CNVs in the normal genome [14]. Furthermore, Genome-wide Association Studies (GWAS) have made important connections between clinical conditions and these common sequence polymorphisms. Here we assessed the genome profile of liver cells of patients with and without steatosis using a very stringent criteria for data analysis (see methods), and we found a considerable variability in CNVs already described in the Database of Genomic Variants, http://projects.tcag.ca/variation/. The limited number of samples included in the present study prevents us from making definite conclusions about association between certain CNV changes and steatosis. However, the differences in the profiles of chromosome regions 3q29, 6p21, 11q11, and 22q11 between patients with and without steatosis are...
remarkable (Fig. 2B), and the study lays a foundation for further investigations of variations potentially associated with the disease. For example, patients with steatosis exhibited lower copy number at chromosome region 3q29 than reference DNA. The MUC20 gene mapping to this region, and represented for more than three consecutive probes in the array, encodes for a protein that interacts with MET impairing the HGF-induced biological effects by attenuating ERK1/2 activation in the Grb2-Ras pathway [15]. Five patients with steatosis showed CNV gains at 22q11, the genome region where GSTT1 maps. Glutathione S-transferases (GSTs) are metabolic enzymes that catalyze conjugation of reduced glutathione (GSH) with a variety of electrophilic compounds including carcinogens, therapeutic drugs and environmental toxins, as well as endogenous substances [16]. Association studies have shown a link between GSTM1 and GSTT1 genotypes with cancer susceptibility and cancer therapy outcome [17,18]. Furthermore, polymorphisms of GSTT1 have been found to be associated with high risk of alcoholic liver diseases [19,20], as well as liver cirrhosis and pancreatitis in individuals with history of alcohol abuse [21]. Apart from these recurrent CNV-changes, we found structural variation in isolated samples from patients with steatosis, including losses at chromosomes 7p21.3 and 10q21.3, and gains on chromosome region 6q16.3, encompassing disease-associated genes. For example, copy number gain at chromosome region 6q16.3 was found in an obese patient (BMI=37.5), involving the Melanin-Concentrating Hormone Receptor 2 gene (MCHR2). This gene encodes for a 19-aa neuropeptide, is expressed almost exclusively within the lateral hypothalamus, and is implicated in regulation of body weight [22]. Interestingly, supportive evidence for a role of MCHR2 in energy balance was derived from a mouse model in which ablation of this gene produced a lean phenotype. Furthermore, the same cytogenetic band has been found involved in a translocation in an obese patients [23].

We also detected genome imbalances at the locus of the Glucosamine-6-Phosphate Deaminase 1 (GNPDA1) in a patient with steatosis (Table 2), and polymorphisms of this gene have been shown to be associated with obesity [24]. Interestingly, a patient who suffered of obstructive biliary lithiasis (Bio119), exhibited SLC10A1 allelic loss. The protein encoded, Na+ taurocholate cotransporting polypeptide (NTCP), participates in the uptake of bile acid conjugated bile salts through the hepatocyte membrane [25,26]. We therefore speculated that the allele imbalance may cause abnormal bile salt clearance and cholestatic liver disease. However, expression analysis demonstrated that the transcriptional level of the gene was higher in liver cells of this patient than in two patients and a HCC cell line (HepG2) with normal SLC10A1 gene dosage. Most likely this is an adaptive response, since expression of this transport protein is regulated by nuclear hormone receptors for which bile salts are specific ligands [27].

5. CONCLUSION

Our array-CGH analysis showed differences in copy number of CNVs of several loci between patients with and without steatosis. The role of chromosomal structural variations in the pathogenesis or the evolution of steatosis cannot be accurately inferred from this small group of patients. However, the study highlights new research directions in this complex disease, and suggests that genomic structural variations may be associated with the pathogenesis or the evolution of steatosis.
CONSENT

Not applicable.

ETHICAL APPROVAL

The approval for the study was provided by the Ethical Committee of the Basque Country Department of Health, and written informed consent was obtained from all patients, according to the Declaration of Helsinki Principles.

ACKNOWLEDGEMENTS

We would like to thank Prof. Paul Edwards for critical reading of the manuscript and BIOEF (Eusko Fundazioa) for samples management and handling. This work was supported by funding from Fondo de Investigación Sanitaria (ISCiii), Grant # 05/1117 to LAP; National Council of Sciences and Technologies (CONICET); Argentine Agency for Science and Technology (ANPCyT–FONCyT: PICT-2011-1897). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. FR is a CIBERehd fellow associated to CIC bioGUNE.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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