Analysis of DNA Changes in the LPL Gene in Patients With Familial Combined Hyperlipidemia

E. Gagné, J. Genest, Jr, H. Zhang, L.A. Clarke, M.R. Hayden

Abstract Familial combined hyperlipidemia (FCHL) is a common lipid disorder characterized by an increase in cholesterol and/or triglyceride levels in multiple individuals of the same family. Prior reports document a decreased activity of lipoprotein lipase (LPL) in FCHL, and studies of the role of LPL in the remodeling of nascent lipoproteins suggest that disturbances in LPL function could underlie FCHL. We studied the LPL gene in 31 unrelated individuals with FCHL. A total of 25 DNA changes (13 "silent" substitutions and 12 DNA changes resulting in amino acid substitutions) were detected in 16 patients. Three new exonic polymorphisms as well as a previously described Ser477→stop and an Asp→Asn substitution were seen with similar frequency on control and FCHL chromosomes. Two novel DNA changes resulting in an Asp209→Val and an His44→Tyr substitution were seen in only two FCHL individuals. In vitro studies showed no effect of these mutations on LPL catalytic activity. LPL mutations impairing catalytic activity did not represent a significant factor leading to FCHL in this population. Variations in any portion of the coding region of the LPL gene affecting other functions besides catalysis are not a frequent cause of FCHL.

Key Words: • molecular genetics • lipoprotein lipase • DNA changes

Familial combined hyperlipidemia (FCHL) is a common genetic disorder in humans, with a population prevalence of 0.5% to 2%, and is seen in approximately 10% of men with premature coronary artery disease. The diagnosis of FCHL is based on ascertainment of multiple individuals in the same family with different types of primary hyperlipidemia including an increase in cholesterol and/or triglyceride levels.

The genetic basis for FCHL has not been determined. Genetic studies indicate that it is due to either a single autosomal dominant gene with variable expression or the combined effect of multiple genes. A characteristic feature of the metabolic disturbance in FCHL is overproduction of hepatic apolipoprotein (apo) B of very-low-density lipoprotein. In addition, decreased lipoprotein lipase (LPL) activity has been shown in one third of the cases of FCHL, which suggests that mutations in the LPL gene in some individuals that lead to partial defects in LPL catalytic activity may be related to the lipid phenotype of FCHL.

Recent studies suggest that LPL is a multifunctional protein with other roles in addition to hydrolysis of triglyceride-rich lipoproteins. Mutations in different domains of the gene might specifically impair certain specific functions of the protein. The relation between the abnormal lipid phenotype of FCHL might not result exclusively from a decreased catalytic action of LPL but also from the role of LPL in the remodeling of nascent lipoproteins in and near the space of Disse in the liver. In this situation, mutations in the LPL gene could result in impairment of LPL function, independent of catalytic activity. This could affect the capacity of LPL to remodel nascent lipoproteins within the liver, resulting in reduced uptake and hence an apparent overproduction of apoB. In this model, the relation between disturbances in LPL function and FCHL would be mediated through domains critical to the function of LPL in affecting uptake of apoB but not necessarily impairing the ability of LPL to function in the catalysis of triglyceride-rich lipoproteins.

Since the original description of mutations underlying LPL deficiency in 1989, over 40 different mutations causing complete deficiency of LPL have been described. The vast majority aggregate in exons 4, 5, and 6 in the regions of the gene showing marked cross-species conservation in LPL. More recently, mutations in the LPL gene associated with partial defects in LPL catalytic activity have been identified. While those exons critical for catalytic activity are now well defined (exons 4, 5, and 6), exons involved in other functions of LPL, such as interaction with the low-density lipoprotein (LDL) receptor-related protein, lipoprotein, interaction with apoC-II, and influencing uptake of LDL, have not been well characterized.

To explore the role of the LPL gene in FCHL, the coding region of the LPL gene was assessed in 31 unrelated French Canadians with the lipid phenotype consistent with the diagnosis of FCHL. Three new exonic polymorphisms were found that do not appear to be associated with FCHL and have no effect on catalytic function. A single previously described mutation in exon 5 (Glu477→Gly) of the LPL gene was discovered that did not demonstrate segregation with FCHL in this fami-
ly.17,18 This LPL mutation is present in approximately one quarter of persons with LPL deficiency in the province of Quebec and occurs with a heterozygous frequency of 1 in 169 in certain regions of the province.17,19 Two new mutations in the LPL gene were identified in exon 2 that occur within a conserved region of LPL across different species but are not associated with any defect in LPL catalytic function. In this population of 31 patients defects in the LPL gene resulting in impairment of LPL catalytic activity did not account for the FCHL phenotype.

Methods

Subjects

A total of 31 probands with FCHL were ascertained in the Lipid Clinic of the Clinical Research Institute of Montréal. All patients were ascertained as being French Canadian based on a three-generation analysis. The hyperlipidemic status for each subject was assigned on the basis of the first lipid level obtained at the Lipid Clinic. Cholesterol and triglyceride levels were measured,20 and plasma apoB concentration was measured with the Berling nephelometer. In all instances the diagnosis of FCHL was made on the basis of an increase in triglyceride levels above the 90th percentile for age and sex as well as the presence of a first-degree relative with one of the above lipid phenotypes that was different from that of the proband (Table 1). The FCHL subjects were not taking any medication known to affect lipid levels before their assessment. To determine the allele frequency of DNA changes, 49 unrelated consecutively ascertained French Canadian patients attending for reasons other than lipid disorders were used as control subjects.

Analysis of the LPL Gene

Human DNA was isolated by using standard methods.11 Polymerase chain reactions (PCRs) for exons 1 through 9 of LPL were performed12,21 with the exception of exon 3, which was amplified by using the primers LPL-80=5'GGTGGGG- TATTTTAAGAAAGCT3' and LPL-81=5’AAACACT-GTTTGGACACATA3' (annealing temperature, 52°C). The PCRs for single-strand conformational polymorphisms (SSCPs) were performed in 50-μL volumes containing 100 ng template DNA, 75 μmol/L dNTPs, 0.25 μCi [α-32P]dCTP (Amersham), 20 pmol/L of each primer, 1.5 mmol/L MgCl₂, 10 μmol/L tris(hydroxymethyl)aminomethane (Tris), pH 8.3, 50 mmol/L KCl, 0.01% gelatin, and 1.25 units Taq DNA polymerase (BRL). PCR product was diluted from 1:50 to 1:100 in 10 mmol/L EDTA and 0.1% sodium dodecyl sulfate (SDS), and 5 μL solution was added to 5 μL loading buffer (95% formamide, 10 mmol/L NaOH, 0.006% xylene cyanol, and bromophenol blue). Samples were denatured at 95°C for 4 minutes and loaded on a nondenaturing polyacrylamide gel using two different gel conditions: (1) 6% polyacrylamide (19:1 acryl/bis) and 5% glycerol in 1 x TBE buffer at room temperature with a constant power of 40 W for 3 to 6 hours and (2) 10% polyacrylamide (19:1 acryl/bis) and 10% glycerol in 1 x TBE at 4°C with a constant power of 20 W for 18 to 24 hours. Gels were dried on blotting paper and exposed to x-ray film for 1 to 10 days.

Exons showing changes in the band pattern by SSCP as compared with a normal control were amplified by PCR, run on a low-melting-point agarose gel (nuSieve GTG), excised from the gel, and purified by using the DNA purification system “Magic PCR Prep” (Promega). The amplified exons were then sequenced either directly by using the “Circum-

| TABLE 1. Summary of Clinical Data on 31 FCHL Patients |
|-----------------|--------|-------|
| Age, y          | 51     | 11    |
| Weight, kg      | 73     | 11    |
| BMI             | 27.1   | 3.1   |
| TC, mmol/L      | 7.63   | 1.32  |
| TG, mmol/L      | 3.67   | 1.58  |
| HDL-C, mmol/L   | 0.97   | 0.26  |
| VLDL-C, mmol/L  | 1.69   | 0.93  |
| LDL-C, mmol/L   | 4.92   | 0.89  |
| ApoB, mg/dL     | 195    | 45    |

FCHL indicates familial combined hyperlipidemia; BMI, body mass index; TC, total cholesterol; TG, triglycerides; HDL-C, high-density lipoprotein cholesterol; VLDL-C, very-low-density lipoprotein cholesterol; LPL-C, low-density lipoprotein cholesterol; and ApoB, apolipoprotein B.

| TABLE 2. Number and Nature of DNA Changes in 31 Patients With FCHL |
|-----------------|--------|-------|
| No. of DNA Changes | No. of Patients | Total No. of Changes |
| 1               | 8      | 8     |
| 2               | 7      | 14    |
| 3               | 1      | 3     |
| Total           | 16 (50%) | 25    |

FCHL indicates familial combined hyperlipidemia. Of the DNA changes, 13 were exonic silent substitutions, and 12 were amino acid substitutions.
TABLE 3. Exonic Polymorphisms in the LPL Gene in FCHL Patients

<table>
<thead>
<tr>
<th>Exon</th>
<th>Base Change</th>
<th>FCHL, n (%)</th>
<th>Control, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>(405)G→A</td>
<td>3 (5)</td>
<td>5 (5)</td>
</tr>
<tr>
<td>4</td>
<td>(435)G→A</td>
<td>4 (6)</td>
<td>10 (10)</td>
</tr>
<tr>
<td>8</td>
<td>(1164)C→A</td>
<td>6 (10)</td>
<td>14 (14)</td>
</tr>
</tbody>
</table>

LPL indicates lipoprotein lipase; FCHL, familial combined hyperlipidemia. There were 62 FCHL alleles and 98 control alleles.

In Vitro Mutagenesis and Expression Studies

A 1.6-kb cDNA fragment containing the entire coding sequence of human LPL was cloned into a dual-function vector (CDM8) for both mutagenesis and expression.2021 In vitro site-directed mutagenesis was performed by using three different primers: (Asn 9) 5'GAAGAGATTTTATCAACATCGAAAG- 3'; (Val 21) 5'CTAAGGACCGTGATTTGCACGC- 3'; and (Tyr 8) 5'TCATTTCAATTACAGCAGCAAAAC3'. Mutant clones were identified by oligonucleotide hybridization and verified by DNA sequencing. Expression phagemids were introduced in COS-1 cells by electroporation20 with the following modifications. The Gene Pulser apparatus (Bio-Rad Laboratories) was set at 240 V/960 μF. After electroporation, the cells were transferred immediately to a 10-cm culture dish containing Dulbecco’s modified Eagle’s medium (high glucose) and 10% fetal calf serum. After 24 hours the medium was removed and replaced with fresh medium containing 7 μl heparin per milliliter. The medium from each dish was collected every 24 hours for 4 days, snap frozen, and maintained at (−70°C) until assayed for mass and activity.

DNA Analysis in Controls

PCR-based methods of detection were established to ascertain in the control group the allele frequency of the various changes detected in our FCHL patients. Detection of the Asp9→Asn substitution was performed by digesting the PCR-amplified exon with Taq I (BRL) for 3 hours at 65°C. The digest was loaded on an 8% polyacrylamide gel and run at 30 mA for 3 hours. An abnormal 58-bp fragment was detectable in the presence of the mutation. Screening of the Asp9→Val substitution was performed by digesting the PCR-amplified exon 2 with the restriction enzyme Mae III (Boehringer Mannheim). The mutation generates a unique Mae III site that produces a 78-bp and a 135-bp fragment that can be seen when run on a 3% agarose gel. The exon 3 polymorphism [(405) G→A] was detected by digesting the PCR-amplified exon 3 with Hae III (BRL). This mutation abolishes an Hae III site and can be detected by the presence of an abnormal 183-bp fragment when run on a 3% agarose gel. The Ser99→stop mutation was identified by digesting the PCR-amplified exon 9 with Mnl I (NEB) and was seen by running the digest on a 3% agarose gel.

Screening for the (435) G→A was performed by oligonucleotide hybridization of amplified DNA. Exon 4 was amplified by PCR, and approximately 50 ng of the DNA was denatured and transferred in duplicate onto a nylon membrane (Hybond N-Plus, Amersham). Oligonucleotides homologous to the normal and mutant sequences were synthesized and end-labeled with [γ-32P]ATP. Hybridization was performed21 with the following modifications. Labeled oligonucleotides were incubated overnight at 65°C and washed several times in buffer 1 (2× saline-sodium phosphate-EDTA [SSPE] and 0.5% SDS) for 10 minutes at room temperature, in buffer 2 (1× SSPE and 0.1% SDS) for 10 minutes at room temperature, and in buffer 3 (0.1× SSPE and 0.1% SDS) for 12 minutes at 49°C.

Measurements of LPL Activity and Mass

LPL lipolytic activity in COS-1 cell medium was determined by using radiolabeled trilinolenate 0.1% oleate lecithin emulsion as substrate. The released fatty acids were extracted and counted in a liquid scintillation counter, and the activity in COS-1 cell medium was expressed as nanomoles free fatty acids released per minute per milliliter. The medium from each dish was collected every 24 hours for 4 days, snap frozen, and maintained at (−70°C) until assayed for mass and activity.

TABLE 4. Exonic Changes Resulting in Amino Acid Substitutions in Patients With FCHL

<table>
<thead>
<tr>
<th>Location of LPL Gene</th>
<th>Nucleotide Change</th>
<th>Codon Change</th>
<th>FCHL, n (%)</th>
<th>Control, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 2</td>
<td>(106)GAC→AAC</td>
<td>Asp9→Asn</td>
<td>3 (5)</td>
<td>8 (8)</td>
</tr>
<tr>
<td>Exon 2</td>
<td>(144)GAC→GTC</td>
<td>Asp21→Val</td>
<td>1 (1.6)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Exon 2</td>
<td>(211)CAC→TAC</td>
<td>His94→Tyr</td>
<td>1 (1.6)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Exon 5</td>
<td>(644)GGG→GAG</td>
<td>Gly184→Glu</td>
<td>1 (1.6)</td>
<td>(0.6)*</td>
</tr>
<tr>
<td>Exon 9</td>
<td>(1421)TCA→TGA</td>
<td>Ser99→stop</td>
<td>6 (10)</td>
<td>10 (10)</td>
</tr>
</tbody>
</table>

FCHL indicates familial combined hyperlipidemia; LPL, lipoprotein lipase. There were 62 FCHL alleles and 98 control alleles.

*See Reference 19.
Amino Acid Substitutions

Twelve SSCP band shifts were due to nucleotide changes that did result in amino acid substitutions in the LPL gene (Table 4). Six patients had a TCA→TGA nucleotide change resulting in an Ser→stop mutation. This was seen on 10% of the FCHL alleles and also seen on 10% of the control alleles. This polymorphism results in a protein that is normally secreted and has normal catalytic activity against long-chain fatty acids.23 A nucleotide change in exon 5 resulted in a G→A transition resulting in the substitution of a glutamine for glycine at position 188. This mutation appears frequently in the French Canadian population.17,19

Three independent SSCP band shifts were seen in exon 2. The first represented a G→A substitution resulting in an Asp→Asn codon change. This was seen in 5% of the FCHL alleles and 8% of control alleles, including those from one homozygote. The high frequency of this substitution in both control and FCHL families indicates that this change is unlikely to be causative of FCHL in these families.
Two previously undescribed substitutions were found in exon 2. The first represented an A→T change that resulted in a substitution of a valine for aspartic acid at residue 21 (nucleotide 144) (Fig 2, top). This was observed in one of the patients with FCHL and was not seen on any of the control chromosomes. Comparison of this residue 21 in LPL from many different species, including humans, chickens, rats, mice, guinea pigs, pigs, cattle, and cats, revealed that aspartic acid at residue 21 of the LPL gene is highly conserved in all species except chickens. In this species the aspartic acid is replaced by glutamic acid, which represents a conservative substitution. The aspartic acid to valine substitution results in a change in charge and represents a substitution not normally seen in LPL of any of these species (Fig 2, bottom).

A C→T change at nucleotide 211 that resulted in a substitution of tyrosine for histidine at residue 44 in one patient with FCHL was not seen on any of the control chromosomes (Fig 3, top). Histidine is also a highly conserved residue at this position in LPL from nine species, and the histidine to tyrosine substitution represents a nonconservative change (Fig 3, bottom).

Effect of the Newly Identified Amino Acid Substitutions in the LPL Gene on Catalytic Function of LPL

Prior reports have documented the functional effects on catalysis of triglyceride-rich particles of the Gly158→Glu mutation and the Ser447→stop. The Gly158→Glu mutation results in a completely catalytically defective LPL protein when present in the homozygous state,18,24,25 whereas the Ser447→stop mutation is seen in the general population and does not impair the capacity of LPL to hydrolyze long-chain fatty acids.23 However, because the effect of the three residue changes found in exon 2 have not previously been
assessed for their effect on catalytic function, in vitro mutagenesis studies were undertaken.

The three LPL mutants in exon 2 yielded detectable mass in the medium from transfected COS-1 cells that was similar to that seen in COS-1 cells transfected with wild-type cDNA (Table 5). In addition, specific activity was also similar to that of the wild type for the Asp$_9$->Asn (0.32 nmol/min per nanogram), the Asp$_{21}$->Val (0.35 nmol/min per nanogram), and the His$_{18}$->Tyr (0.31 nmol/min per nanogram) substitutions. Consequently, none of these mutations significantly influenced the catalytic activity of LPL.

Population and Segregation Studies

To further define whether any of the DNA changes found were related to the phenotype of FCHL, the frequency of these DNA changes in patients with FCHL were compared with their frequency on normal control chromosomes from subjects of French Canadian descent. The Ser$_{14}$->stop and Asp$_9$->Asn substitutions (Fig 4) were seen on control chromosomes at equal or greater frequency than on chromosomes from patients with FCHL (Table 4). The His$_{18}$->Tyr and Asp$_{21}$->Val changes were not seen on any of the control chromosomes. The frequency of the Gly$_{188}$->Glu substitution is relatively high (1 in 169 individuals) in persons living around Montreal. Further segregation analysis in this family demonstrated that this particular mutation was not segregating with the lipid phenotype of FCHL in this family. This suggested that the Gly$_{188}$->Glu as seen in this FCHL patient was not causative but rather represented the finding of a coincidental DNA change in a population with a high frequency of this mutation (Table 4).

Asp$_{21}$->Val and His$_{18}$->Tyr represent two previously unreported mutations in the LPL gene that were not found on any of the control chromosomes. DNA and lipid analyses were undertaken in family members of both probands. In one family (His$_{18}$->Tyr) the lipid phenotype of FCHL segregated together with the mutation in two siblings. However, only a small number of meioses could be examined (Fig 5). In family members of the proband with the Asp$_{21}$->Val mutation, 4 family members could be ascertained, and in 2 of these with the mutation a normal lipid profile was detected (Fig 5). However, these relatives were at least 10 years younger and weighed 16 kg less than the proband with the DNA change and the lipid phenotype of FCHL.

Discussion

Prior studies of patients with FCHL have identified a subset (36%) who clearly had a deficiency in catalytic activity for LPL. Thus, we searched for changes in the LPL gene that might result in a catalytically defective LPL protein and be responsible for the FCHL phenotype in our 31 patients. A total of 25 DNA changes in 16 patients of this cohort were detected.

Three previously undescribed exonic polymorphisms in the LPL gene were defined in exons 3, 4, and 8 that resulted in nucleotide changes. However, these changes, which accounted for 13 of the DNA changes, had no effect on the amino acid composition of the protein. Amino acid substitutions represented the remaining 12 DNA changes detected. In 6 patients a nucleotide substitution (TCA->TGA) resulted in a stop mutation and a truncated protein that had an allele frequency that was similar to that of persons in the general population of the same ancestry. In 3 patients a previously reported Asp$_9$->Asn substitution was detected that was seen with slightly higher frequency in the control population and therefore cannot be invoked as a cause for the FCHL phenotype. A Gly$_{188}$->Glu mutation was found in 1 patient with FCHL, but this did not occur at a greater frequency than what would be expected on normal French Canadian chromosomes and did not segregate with the phenotype of FCHL in this particular family.

Two other mutations in exon 2 (Asp$_{21}$->Val and His$_{18}$->Tyr) were seen only on FCHL chromosomes. In vitro mutagenesis studies indicated that these mutations have no effect on the catalytic function of the LPL.

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**TABLE 5. LPL Activity, Mass, and Specific Activity in COS1 Cell Medium**

<table>
<thead>
<tr>
<th>cDNA</th>
<th>Mass, ng/mL (% Wild Type)</th>
<th>Activity, per Milliliter (% Wild Type)</th>
<th>Specific Activity, nmol/FFA/min per Nanogram</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type LPL</td>
<td>369 (100)</td>
<td>104 (100)</td>
<td>0.28</td>
</tr>
<tr>
<td>Asp$_9$-&gt;Asn</td>
<td>304 (82)</td>
<td>96 (92)</td>
<td>0.32</td>
</tr>
<tr>
<td>Asp$_{21}$-&gt;Val</td>
<td>357 (97)</td>
<td>124 (119)</td>
<td>0.35</td>
</tr>
<tr>
<td>His$_{18}$-&gt;Tyr</td>
<td>459 (125)</td>
<td>144 (138)</td>
<td>0.31</td>
</tr>
</tbody>
</table>

LPL indicates lipoprotein lipase; FFA, free fatty acid.

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**Fig 4.** Schematic representation of how a G->A substitution at nucleotide 106 in exon 2 abolished a Taq I restriction site. The proband for familial combined hyperlipidemia (lane 13) is heterozygous for the Asp$_9$->Asn substitution with a normal (52-bp) and an abnormal (58-bp) band. Lanes 1 through 12 are controls; lane 1 represents a homozygote and lane 4, a heterozygote. PCR indicates polymerase chain reaction.
protein. We therefore concluded that in this particular cohort none of these DNA changes could account for a decreased LPL activity and, consequently, an FCHL lipid phenotype. Mutations in the LPL gene that result in a catalytically defective protein are likely to be uncommon causes of FCHL in this particular population. In addition, this study clearly showed that exonic changes in the LPL gene are frequent (13/62 alleles, or 20%) and further supports the need for functional studies to allow assessment of any DNA changes in the LPL gene.

The lipid phenotype of FCHL, however, may be related to LPL not through mutations affecting catalytic activity but rather through DNA changes affecting other domains of the protein that may play a role in the remodeling of nascent lipoproteins in the liver. In this regard two mutations in exon 2 of this gene are of interest. These mutations occur in residues that are conserved across LPL of nine different species. Further studies are clearly indicated to assess the affect of these changes in the LPL gene. The family studies thus far are insufficient to either clearly support or refute the hypothesis that these DNA changes are associated in some way with the phenotype of FCHL. The presence of normal lipid profiles in the proband’s sibling and daughter, both of whom have the Asp21 Val mutation similar to the proband, may suggest that this DNA change is not related to the phenotype of FCHL in this family. However, the sibling is 10 years younger and the daughter 20 years younger than the proband. Unlike the proband, they have a normal body mass index, and it could also be argued that the environmental factors interacting with this DNA change are not yet operative. Further extended family studies are needed to resolve this issue.

The phenotypic heterogeneity of FCHL may be due to mutations in different genes that might be interacting. Patients with mutations in the LPL gene that result in partial deficiency of LPL activity may have significant lipid disturbances, primarily chylomicronemia, when exposed to environmental stresses such as pregnancy, diabetes, or alcohol. In addition, some patients may be particularly susceptible to these "triggers" if they also have other genetic factors, such as an apoE-2 allele, that might affect the uptake of remnant particles. The patient with the His44 Tyr mutation had an E3/E3 genotype, while the proband with the Asp21 Val substitution had an E3/E4 genotype. Thus, it would appear that in these FCHL patients there was no association with the apoE-2 isoform in contrast to that in patients with environmentally induced chylomicronemia. In this situation patients who had mutations only partially affecting catalytic activity of the LPL gene were more likely to manifest with significant lipid disturbances in the presence of an apoE-2 genotype.

While the biochemical phenotype associated with FCHL is associated with hyperapoB, the genetic defect underlying the increase in apoB levels has not yet been defined. This study demonstrated that defects in the LPL gene that could result in impairment of catalytic activity do not represent significant causes of FCHL in this particular population. However, this study has not completely excluded the role of variation in the coding region of LPL in the causation of the lipid phenotype of FCHL that may be mediated by domains independent of the catalytic function of LPL. However, if this occurs, it would represent an infrequent contribution to the presentation of FCHL.

Acknowledgments

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References


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