Identification of a genetic marker of Helicobacter pylori strains involved in gastric extranodal marginal zone B cell lymphoma of the MALT-type

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Background and aims: Gastric extranodal marginal zone B cell lymphoma of the mucosa associated lymphoid tissue (MALT)-type (MZBL) is a rare complication of Helicobacter pylori infection. Currently, no bacterial factor has been associated with the development of this disease. Our aim was to identify genes associated with lymphoma development.

Methods: We used subtractive hybridisation as a tool for comparative genomics between H pylori strains isolated from a patient with gastric MZBL and from a patient with gastritis only.

Results: When gastric MZBL strains were compared with gastritis strains, two open reading frames (ORFs) were significantly associated with gastric MZBL: JHP950 (74.4% vs 48.7%, respectively; p = 0.023) and JHP1462 (25.6% vs 2.6%, respectively; p = 0.004). The prevalence of JHP950 was 48.8% (p = 0.024) in duodenal ulcer strains and 39.3% (p = 0.006) in gastric adenocarcinoma strains, which makes this ORF a specific marker for gastric MZBL strains. In contrast, the prevalence of JHP1462 was 16% (p = 0.545) and 35.7% (p = 0.429) in duodenal ulcer and adenocarcinoma strains, respectively. These ORFs were present in reference strain J99 but not in reference strain 26695. JHP950 is located in the plasticity zone whereas the other, JHP1462, is located outside. Both encode for H pylori putative proteins with unknown functions.

Conclusion: Despite its low prevalence, the ORF JHP1462 can be considered a candidate marker for H pylori strains involved in severe gastroduodenal diseases. In contrast, the ORF JHP950 has a high prevalence, and is the first candidate marker for strains giving rise to an increased risk of gastric MZBL strains. Further confirmation in other studies is needed.
To evaluate the prevalence of the open reading frames (ORFs) identified from the “tester” strain in the subtractive library, a collection of 43 H pylori strains obtained from gastric MZBL patients (27 men and 16 women, mean age 48.2 (13.4) years) were used. These strains were isolated from patients included in two French multicentre studies by the Groupe d’Etude des Lymphomes de l’Adulte (GELA). 92 In d’Etude Franc¸ais des Lymphomes Digestifs (GELD) of the included in two French multicentre studies by the Groupe years were used. These strains were isolated from patients in a library, a collection of 43 (ORFs) identified from the “tester” strain in the subtractive "on".

kanamycin and grown overnight at 37˚C under agitation. Ant bacteria were inoculated into LB media with 50 mg/l. Th threshold cycles was observed between subtracted and unsubtracted products, indicating an important reduction of subtractive hybridisation was evaluated using amplification of a fragment of H pylori 16S rDNA in a real time PCR experiment in a LightCycler thermocycler (Roche Diagnostics, Palo Alto, California, USA) was used with the “tester” (gastric MZBL) and “driver” (gastritis) strains. The manufacturer’s recommendations were followed with the exception of the choice of the restriction enzyme and the method of evaluation of the subtractive efficacy. The restriction enzyme AflI was chosen because of its ability to generate DNA fragments, from “tester” and “driver” strains, in the same molecular weight range (from 2.5 kb to 0.25 kb). The efficacy of subtractive hybridisation was evaluated using amplification of a fragment of H pylori 16S rDNA in a real time PCR experiment in a LightCycler thermocycler (Roche Diagnostics, Neully sur Seine, France). Results were plotted versus time, represented by cycle number, to produce a measure of PCR amplification at the end of each PCR cycle. A difference of 20 threshold cycles was observed between subtracted and unsubtracted products, indicating an important reduction in 16S rDNA hybridisation in the subtracted sample and therefore very good subtractive hybridisation efficacy (data not shown).

After the last step, the subtractive experiment products were cloned in the pTAdv plasmid (Clontech, Palo Alto, California, USA) according to the manufacturer’s recommendations. A total of 139 recombinant bacteria were inoculated into LB medium with 50 mg/l kanamycin and grown overnight at 37°C under agitation. Inserts were amplified by PCR using the following protocol: 1 ml of incubated LB medium was heated for 10 minutes at 95°C and then centrifuged for five minutes at 14 000 rpm. The boiling lysis supernatants were collected and the insert size from each clone of the library was determined by PCR amplification using 5 µl of the supernatant and vector based primers. The average sizes of subtracted fragments varied from 300 to 1850 bp, in agreement with the initial digestive DNA library. DNA fragments were purified using Microspin S-400 HR columns (Amersham Pharmacia Biotech Inc., Uppsal, Sweden), and direct sequencing was performed with the ABI PRISM BigDye Terminators v3.0 Cycle Sequencing Kit (PE Applied Biosystems, Foster City, California, USA) using an ABI 3700 Analyzer DNA Sequencer (PE Applied Biosystems).

Screening of the subtractive library
To determine if the subtractive clones were really “tester” specific, primers for each clone were designed using the conserved sequence between the sequence identified and the corresponding gene of H pylori in database sequences (http://www.probes.toulouse.inra.fr/multilatin/multilatin.html). The specificity of our primers was tested on both H pylori reference DNAs. For the sequences with no homology found in the databases, the specificity of the amplified fragment was determined by direct sequencing and dot blot hybridisation between “tester” (gastric MZBL) and “driver” (gastritis) DNAs, as described below.

If a PCR product was amplified from the “tester” DNA and not from the “driver” DNA, the sequence was considered to be “tester” specific. PCR amplifications were carried out in a 25 µl volume containing 2.5 µl of 10× PCR buffer (Eurobio, Les Ulis, France), 1.5 mM MgCl2 (Eurobio), 200 µM (each) of the deoxynucleoside triphosphates (Eurobio), 2 U of Taq DNA polymerase (Eurobio), 1 µM (each) of the primers (Q BIOgen, Strasbourg, France) and 10 ng of H pylori DNA. After four minutes of initial denaturation at 94°C, each reaction mixture was amplified for 35 cycles as follows: 30 seconds at 94°C, 30 seconds of annealing at 60°C, and extension at 72°C (the time depending on the length of the sequence to be amplified and the processivity of the Taq polymerase). After the last cycle, an extension was continued for another 7–10 minutes. PCR products were separated electrophoretically on agarose gels and stained with ethidium bromide.

Dot blot hybridisation
Screening of the PCR sequences specific for the “tester” strain was performed using our collection of H pylori gastric MZBL and gastritis strains. Dot blot analyses were carried out using the same batch of membranes, prepared with the same dilution of DNA. Genomic DNA (100 ng) of each strain (MZBL, gastritis, J99, and 26695 reference strains) were spotted on a Hybond N+ membrane (Amersham Pharmacia Biotech) by means of a Bio-Rad dot blot 96 well filtration system apparatus (Bio-Rad, Ivry-Sur-Seine, France). Hybridisation was performed using digoxigenin labelled probes of each ORF identified, as described previously. The probes were prepared by PCR after primer design in a conserved area between the “tester” DNA sequence and the J99 reference strain or between both the J99, 26695, and “tester” strain when the DNA sequence was present in all.

Reverse transcription-PCR
RNA was extracted from the “tester” strain and the reference strain J99 during the exponential growth phase using the RNAeasy kit (Qiagen). RNA samples were treated with Rnase-Free Dnase (Promega, Madison, Wisconsin, USA) and quantified spectrometrically. Each reverse transcription PCR (RT-PCR) was carried out in a 20 µl volume containing 250 ng of RNA template, 0.6 µM of each oligonucleotide primer, 0.125 µl of RNAsine (Promega), 2 µl of 5× Q buffer, and 2 µl of RT-Taq mix. Complementary DNA was synthesised at 45°C for 30 minutes followed by 95°C heat inactivation of the RT Taq polymerase for 15 minutes, and then by the amplification program used for PCR amplification. Absence of DNA in RNA preparations was verified by omitting the RT amplification step. After 40 PCR cycles, products were separated electrophoretically on agarose gels.
Statistical analysis

Statistical analysis was performed using STATA 7.0 statistical software (Stata Corporation, College Station, Texas, USA). A first comparison of the characteristics between MZBL and gastritis strains was performed using Fisher’s exact test.

In order to create a variable describing how the different strain characteristics were associated, a multiple correspondence analysis (MCA) was performed followed by a cluster analysis using the average aggregation method and Euclidian distance. Both methods used the Statbox 2.5 program (Grimmer Logiciels, Paris, France).

The odds of an association with MZBL and its 95% confidence interval (CI) were compared within groups of strains and between the different strata of the summary variables obtained by the MCA.

RESULTS

PCR analysis of the subtractive clones

We sequenced the inserts of 132 clones on both strands, of which 81 were non-redundant. PCR screening between “tester” (gastric MZBL) and “driver” (gastritis) DNAs of these 81 clones showed that 39 (48.2%) contained sequences which were specific to the “tester” strain used in the experiment. Homologies of these clones with the \textit{H. pylori} databases (http://www.tigrblast.tigr.org/cmr-blast) and the NCBI blast program (http://www.ncbi.nlm.nih.gov/BLAST) were analysed. The following classification was made: 26 matched with sequences from the two reference strains (J99 and 26695), 10 matched with only J99 specific sequences, none matched with only strain 26695, and three presented no homology with \textit{H. pylori} reference strains.

For 10 of the 26 sequences which matched with the two reference strains, PCR products obtained from the “tester” DNA were different from the expected size when compared with the “driver” DNA and \textit{H. pylori} J99 and 26695 reference DNAs. Direct sequencing of these products showed significant variations, such as insertions or deletions, and therefore they were not included in the dot blot screening of our strain collections to avoid impairing the analysis. Nine other sequences where variation corresponded to intergenic regions were also not tested. The seven remaining sequences (clones...
Dot blot analysis of the selected clones (MALT v gastritis)
The distribution of the 24 selected sequences was tested by
dot blot using our collection of *H pylori* gastric MZBL and
gastritis strains. The specificity of the sequences found to be
“tester” specific by PCR was verified by (1) the absence
of hybridisation on the plot corresponding to the “driver” strain
and (2) the absence of hybridisation for the original
sequences on the plots corresponding to the “driver” DNA,
strain J99, and strain 26695. Indeed, negative results by PCR
may be due to either an absence of the tested gene (true
negative) or a lack of primer annealing due to interstrain
variation in the sequences targeted by the primers.

The 21 remaining sequences were then tested. The corres-
ponding genes and results obtained are summarised in table 1.
The proportion of each ORF was compared between strains
having these three markers, 10 (90.9%) corresponded
to gastric MZBL strains (p = 0.018) (fig 2).

Multiple correspondence analysis
Firstly, an MCA was performed for all variables obtained from
dot blot. We were unable to identify gene clusters which were
significantly associated with gastric MZBL or gastritis strains.
Thus the analysis was focused on the two ORFs found
associated with the gastric MZBL strains. Because the
prevalence of JHP1462 in gastric MZBL and gastritis strains
was low, it was impossible to keep it in our final analysis. In
contrast, the ORF JHP950 was included in the data from our
previous study in which we identified a gene cluster in gastric
MZBL strains, which is absent in gastritis strains, and
containing *iceA1*, *sabA* “on”, and *barZ* “off”. Interestingly,
the dendrogram obtained from the results of the cluster
analysis of the *H pylori* gastric MZBL strain population showed
that the ORF JHP950 was included in the formally identified
gene cluster (data not shown). The possible association
between the presence of gastric MZBL and the characteristics
of strains cultured from these patients was tested. On analysis
of the summary variables according to the MCA results, it was
remarkable that the odds of having gastric MZBL among
patients harbouring JHP950, *iceA1*, and *sabA* “on” strains were
10 times higher than for the other defined groups (odds ratio
10.3 (95% CI 1.2–86.0)) (table 2); indeed among a total of 11
strains having these three markers, 10 (90.9%) corresponded
to gastric MZBL strains (p = 0.018) (fig 2).

Verification of the specificity of the two putative
markers
The specificity of the association of the ORFs JHP950 and
JHP1462 with gastric MZBL strains was evaluated by
determining their prevalence in a collection of duodenal
ulcer strains (n = 41) by dot blot analysis, as described above.
The prevalence in duodenal ulcer strains of ORFs JHP950 and
JHP1462 was 48.8% (p = 0.024) and 16% (p = 0.545),
respectively.

The prevalence of ORFs JHP950 and JHP1462 on strains
isolated from 28 gastric adenocarcinoma was also deter-
mined. As DNA obtained from these strains was not
concentrated enough to perform dot blot analysis, PCR
screening was performed with the primers used for dot blot
probe synthesis. A prevalence of 39.3% (p = 0.006) for
JHP950 and of 35.7% (p = 0.429) for JHP1462 ORFs was
found (table 3).

These results confirm that JHP950 is a specific marker for
*H pylori* strains isolated from gastric MZBL (table 3).

**DISCUSSION**
Following sequencing of the whole genome of two strains of
*H pylori*, the possibility arose to study the genetic diversity of

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**Table 2** Strength of the association of *Helicobacter pylori* genotypes and extranodal marginal zone B cell lymphoma of the MALT-type (gastric MZBL) status versus gastritis status

<table>
<thead>
<tr>
<th></th>
<th>Total No of patients</th>
<th>Patients with MZBL</th>
<th>Patients with gastritis</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>JHP950</strong></td>
<td>82</td>
<td>43</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>31</td>
<td>11</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>51</td>
<td>32</td>
<td>19</td>
<td>3.1 (1.2–7.8)</td>
</tr>
<tr>
<td><strong>MCA summary variables</strong></td>
<td>70</td>
<td>39</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td>59</td>
<td>29</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>iceA1, sabA “on”, and JHP950+</td>
<td>11</td>
<td>10</td>
<td>1</td>
<td>10.3 (1.2–86.0)</td>
</tr>
</tbody>
</table>

OR, odds ratio, presented as univariate results; CI, exact binomial 95% confidence interval; MCA, multiple correspondence analysis.

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![Figure 2](http://www.gutjnl.com)
Subtractive hybridisation was later validated by *H pylori* of the hybridisation, led to the discovery of the known. Our hypothesis was that tempting but limits the possibilities to the genes already clonal group of genomic differences have been identified in a virulent strain interpatient transmission and colonisation. For example, studies performed previously compared bacterial strains with genovar III strains. Concerning *cepacia* *pestis* highly specific detection systems: for example, in specific sequences which could provide a basis for developing bacteria from different species in order to identify nucleotide Subtractive hybridisation has also been used to compare *H pylori* strains using DNA arrays. Indeed, this approach is now considered an important tool for comparative prokaryotic genomics. Most of the studies performed previously compared bacterial strains with apparent enhanced capacity for human infection and/or interpatient transmission and colonisation. For example, genomic differences have been identified in a virulent strain of *Klebsiella pneumoniae* as well as in the uropathogenic *Escherichia coli* strain 536 compared with the non-pathogenic strain MG1655, and more recently a genomic locus was identified and characterised in the Brazilian pupuric fever clonal group of *Haemophilus influenzae* biogroup *aegyptius*.11–13 Subtractive hybridisation has also been used to compare bacteria from different species in order to identify nucleotide specific sequences which could provide a basis for developing highly specific detection systems: for example, in *Yersinia pestis, Salmonella enterica* serovar Enteritidis, or *Burkholderia cepacia* genovar III strains.14–16 Concerning *H pylori*, representation of differences analysis, a method similar to subtractive hybridisation, led to the to discovery of the *cag* pathogenicity island.17 Subtractive hybridisation was later validated by Akopyants et al and allowed identification of a 2 kb transposable element named IS607, as well as more recently a new type IV secretion system present in the plasticity zone of the *H pylori* genome.18–20

In a subtractive hybridisation experiment, the choice of strains to be compared is particularly important. Considering the fact that the natural evolution of *H pylori* infection begins with gastritis and moreover that host susceptibility factors of developing gastric MZBL are currently unknown, this choice is especially difficult. We based our choice on the pathology (gastric MZBL versus gastritis) and age of the patients from whom the strains were isolated (29 year old patient with gastric MZBL, 74 year old patient suffering from gastritis). The advanced age of the “driver” strain patient was chosen to minimise the possibility that gastritis would evolve to more severe gastrointestinal disease. As the mean age of patients suffering from gastric MZBL is close to 50 years, we could anticipate a particular pathogenic property in the tester strain issued from a much younger patient. Furthermore, the two strains were chosen because of their common profile for six known *H pylori* virulence genes—that is, *cagA*, *cagE*, *vacA*1m1, *babA2*, *iceA1*, and *tphA* “on”, and one adhesin *sabA* “on”.

MCA analysis, initially carried out on all of our dot blot data, failed to identify a gene cluster associated with gastric MZBL strains. However, despite the fact that our collection of gastric MZBL strains is the largest of those published, the number of strains included in this study may not be sufficient to efficiently perform MCA.

The first category of sequences identified presented significant homologies with genes present in strains 399 and 26695 but with important deletions or insertions. Such variations can modify the functional status of a gene or the functionality of the deduced protein. Moreover, it has been suggested that mixtures of matching and divergent sequences can facilitate quite dramatic changes in bacterial phenotype. We originally expected to find new *H pylori* genes and indeed we identified three sequences of unknown genomic localisation, corresponding to new putative coding regions. Nevertheless, none of these sequences was significantly associated with gastric MZBL. Such results could not have been found using a standard approach for detecting genetic diversity, and thus our findings provide more information for the future in investigating the particular properties of these strains.

The results of the dot blot experiments (MALT versus gastritis) highlighted two ORFs (JHP950 and JHP1462). Globally, repartition of both ORFs (JHP950 and JHP1462) was statistically different (p = 0.016 and p = 0.002, respectively) when comparing the four *H pylori* strain collections studied.

The prevalence of ORF JHP1462 (table 3) in MZBL strains was indeed low (25.6%) but when compared with gastritis strains the difference was highly significant (p = 0.004). However, no significant difference was found between gastric MZBL strains and duodenal ulcer and gastric adenocarcinoma strains (table 3), indicating that JHP1462 is not a specific genetic marker for *H pylori* gastric MZBL strains but could be considered more generally as a candidate marker for *H pylori* strains associated with severe gastroduodenal diseases. It is located outside the plasticity zone, in common with two thirds of *H pylori* strain specific genes.

Table 3 Prevalence of JHP950 and JHP1462 open reading frames (ORFs) in *Helicobacter pylori* strains isolated from patients with gastritis, duodenal ulcer, and gastric adenocarcinoma in comparison with strains isolated from patients with gastric MZBL

<table>
<thead>
<tr>
<th>Gastritis</th>
<th>Duodenal ulcer</th>
<th>Gastric adenocarcinoma</th>
<th>Gastric MZBL</th>
</tr>
</thead>
<tbody>
<tr>
<td>JHP950</td>
<td>48.7 (p = 0.023)*</td>
<td>48.8 (p = 0.024)*</td>
<td>39.3 (p = 0.006)*</td>
</tr>
<tr>
<td>JHP1462</td>
<td>2.6 (p = 0.004)*</td>
<td>16.0 (p = 0.545)</td>
<td>35.7 (p = 0.429)</td>
</tr>
</tbody>
</table>

MZBL, marginal zone B cell lymphoma of the MALT-type.

*p values were determined by Fisher’s exact test.

*Significant values versus gastric MZBL strains.

In contrast, the prevalence of the JHP950 ORF in gastric MZBL strains was significantly higher than in strains isolated from duodenal ulcer and gastric adenocarcinoma. The difference in prevalence for this marker was in the same range as the difference between the prevalence of *cagA* in duodenal ulcer and gastric adenocarcinoma strains compared with gastritis strains. Indeed, in the past we looked for the *cagA* gene in relation to clinical presentation in a collection of 167 *H pylori* strains. The *cagA* gene was found in 82.6% of duodenal ulcer strains versus 63.3% of gastritis strains.37

These results were confirmed later in several European studies.43–46 The *cagA* gene was first considered as a marker of virulence for *H pylori*. Indeed, following the discovery of the *cagA* pathogenicity island and of the interaction of the *cagA* protein with the epithelial cell, it is now considered to be a true virulence factor associated with severe clinical outcome (that is, duodenal ulceration and gastric adenocarcinoma).47–50 Similarly, the ORF JHP950 is part of the plasticity zone of strain J99. This region is approximately 45 kb long in strain J99 and 68 kb long in strain 26695, and contains one third of the strain specific genes. Occhialini et al studied several ORFs in the plasticity region of strain J99 by hybridisation and found that most were functional.51 We verified by RT-PCR that JHP950 and JHP1462 were transcribed. They were originally annotated as *H pylori* specific proteins with unknown functions (that is, they have no orthologs in other bacterial sequences). This classification is
still accurate after publication of a revised annotation and comparative analysis of \textit{H pylori} genomes.\textsuperscript{44} Our results are in line with the study of Salama et al who found that the most important class of strain specific genes was more frequent among strain specific genes (58\%) than in the core of the genome (21\%) which contains genes encoding, for example, metabolic functions.\textsuperscript{21}

A limit of our study was the use of PCR instead of hybridisation for detection of the ORFs JHP950 and JHP1462 in adenocarcinoma strains. Indeed, there are very few collections of \textit{H pylori} strains obtained from patients with gastric adenocarcinoma in Europe. The DNA material obtained did not allow us to perform dot blot hybridisation but only detection by PCR. The latter technique can have false negative results because of a lack of primer binding due to limited differences in sequences. Therefore, we tested the accuracy of our method on our strain collections and found a sensitivity of 90\% (JHP950) and 95\% (JHP1462) versus dot blot hybridisation, which confirms the relevance of our approach.

MCA analysis allowed us to show that the JHP950 marker is part of a gene cluster significantly associated with gastric MZBL strains. This gene cluster, which is comprised of JHP950, \textit{ice}A1 alleles, and a \textit{sa}B \textit{on} status, partially validates our initial findings.\textsuperscript{13} The \textit{sa}B gene encodes for an adhesin. The association of JHP950 with \textit{ice}A1, a gene activated by contact with the gastric epithelium, and an adhesin seems to be in line with the function of these genes.\textsuperscript{45,50} This underlines the fact that JHP950 might have functional importance during interaction of the bacterium with cells. We can hypothesise that if JHP950 corresponds to a new \textit{H pylori} virulence factor, \textit{H pylori} strains harbouring this triple association of genes could have a pathogenic advantage. Indeed, the odds of having gastric MZBL among patients harbouring JHP950, \textit{ice}A1, and \textit{sa}B \textit{on} strains were 10 times higher than for the other defined groups. Koehler et al recently reported that the \textit{vac}A m2 allele was the predominant subtype in MALT lymphoma.\textsuperscript{22} We also previously reported a slight association between \textit{m}2 genotype and MALT strains (when considering the \textit{m} genotype only) but we found no association between \textit{vac}A \textit{s}m2 alleles and \textit{ice}A1 genotypes.\textsuperscript{10} However, we share the similar conclusion (that is, certain \textit{H pylori} subtype combinations have a predictive value for the development of gastric MZBL).

In conclusion, if JHP1462 cannot be considered a specific genetic marker of gastric MZBL strains but rather as a new candidate marker of \textit{H pylori} virulent strains in general, in contrast JHP950 can be considered as the first candidate marker of gastric MZBL \textit{H pylori} strains. The association of JHP950 with gastric MZBL strains is similar to that between \textit{cag}A and duodenal ulcer strains. Moreover, JHP950 forms a gene cluster with \textit{ice}A1 and \textit{sa}B \textit{on} which is associated with gastric MZBL strains. Future studies are needed to confirm this association and to understand the biological function of this ORF in order to gain insight into the pathogenesis of \textit{H pylori} and the development of gastric MZBL.

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REFERENCES

EDITOR’S QUIZ: GI SNAPSHOT

A painful liver

Robin Spiller, Editor

Clinical presentation

A 62 year old woman was admitted to Elche University General Hospital for further evaluation of an intermittent right upper quadrant pain unrelated to meals or posture. She had previously been well with no history of liver disease, alcohol abuse, or abdominal trauma. On physical examination, there were no signs of chronic liver disease or portal hypertension. Results of laboratory studies, liver function tests, and ultrasound guided liver biopsy were also normal. Real time and colour Doppler ultrasonography showed an anechoic mass-like lesion at the porta hepatis with continuous non-pulsatile flow within the lesion. The gallbladder was normal with no gallstones visualized. Then, a helical computed tomography (CT) scan was performed.

Question

What does this investigation show (fig 1).

See page 943 for answer

This case is submitted by:

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A painful liver

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