Nucleolar organiser regions in normal, cirrhotic, and carcinomatous livers

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SUMMARY A series of 54 liver biopsy specimens was studied by means of the argyrophil (AgNOR) technique for nucleolar organiser region (NOR)-associated proteins. These included normal livers and livers affected by chronic active hepatitis, cirrhosis, hepatocellular carcinoma and adenoma. Four of the cases of cirrhosis showed liver cell dysplasia. The mean numbers of NOR sites in normal, cirrhotic, and carcinomatous livers were significantly different: adenoma had similar mean counts to those in chronic active hepatitis (CAH). There was no overlap between the ranges of NOR counts in normal, cirrhotic, and malignant liver specimens. Where cirrhosis and hepatocellular carcinoma were present in the same specimen, the AgNOR counts were higher in the carcinomatous than cirrhotic areas. To investigate the prospective value of the method a further seven biopsy specimens were studied; in these it had not been possible to decide on a diagnosis between normality and cirrhosis or cirrhosis and hepatocellular carcinoma. In all seven specimens a repeat biopsy or necropsy gave results as predicted by AgNOR staining. It is therefore proposed that quantitation of staining for NOR-associated proteins is a diagnostically useful method in liver disease.

The diagnostic value of counting nucleolar organiser regions (NORs) in cases of malignancy has been emphasised recently. The demonstration of NORs by virtue of the argyrophilia of their associated proteins (NORAPs) is simple, rapid, and reproducible and has afforded useful data in a series of neoplasms, including those of the lymphoid system, pleural mesothelium, breast, small round cell tumours and fibroblastic tumours of childhood, melanocytic tumours, and other skin neoplasms.

In liver disease the distinction between normality, hepatitis, cirrhosis and primary malignancy may at times be difficult. As the cirrhotic process is one of increased cellular activity and because NOR numbers reflect such a process, we examined the NOR count in a series of liver specimens of known pathology, including established cirrhosis, chronic active hepatitis, and hepatocellular carcinoma, and compared these with biopsy specimens of normal livers. Furthermore, a prospective series of equivocal biopsy specimens was examined and the numbers of NORs related to the histological outcome in subsequent material.

Material and methods

Liver biopsy specimens
Fifty four specimens of known liver pathology were examined initially. All were needle biopsies and comprised 10 specimens of normal liver, 10 with chronic active hepatitis (CAH), 10 with cirrhosis (seven established and three in the active phase), and 10 with hepatocellular carcinoma (three “scirrhous”, two “pseudoglandular,” and five “trabecular” types). A further five specimens included concomitant cirrhosis and hepatocellular carcinoma, and four showed liver cell dysplasia in cirrhosis with increased mitoses and nuclear pleomorphism. Finally, five specimens were of liver cell adenoma (table 1). A further seven specimens of equivocal histology were studied. In two of these it was uncertain whether the liver was normal or cirrhotic and in five whether it was cirrhotic or malignant (table 2).

AGNOR technique and enumeration procedure
The one step argyrophil method was used as described previously. This was run at room temperature in the dark for 30–35 minutes. The AgNOR sites were counted in 200 randomly selected hepatocytes, using a ×100 oil-immersion objective and an eyepiece graticule to prevent recounting. Bile ductular, vascular, inflammatory and Kupffer cells were not included in the enumeration procedure. In the cases of cirrhosis and hepatocellular carcinoma 200 hepatocytes were counted from both areas. In the case of bi- or multinucleate cells the numbers of NORs were
Table 1  Mean numbers of nuclear AgNOR sites for each specimen

<table>
<thead>
<tr>
<th>Case No</th>
<th>Normal liver</th>
<th>Chronic active hepatitis</th>
<th>Cirrhosis</th>
<th>Adenomas</th>
<th>Mixed</th>
<th>Hepatocellular carcinoma</th>
<th>Cirrhosis</th>
<th>Hepatocellular carcinoma</th>
<th>Cirrhosis with dysplasia</th>
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<td>15-1</td>
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Mean 1.88  2.12  3.21  2.3  7.88  3.32  7.46  4.48
SD   0.244  0.029  0.33  0.1  2.9  0.39  1.76  0.99
SEM  0.074  0.0078  0.04  0.04  0.88  0.17  0.78  0.495

Table 2  Outcome of a series of seven biopsy specimens of equivocal histology, where AgNOR counts were performed

<table>
<thead>
<tr>
<th>Case No</th>
<th>Diagnostic problem</th>
<th>Mean AgNOR count</th>
<th>Outcome</th>
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<tr>
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<td>*Cirrhosis</td>
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<td>7-1</td>
<td>†Hepatocellular carcinoma</td>
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<tr>
<td>7</td>
<td>?cirrhosis/hepatocellular carcinoma</td>
<td>9-3</td>
<td>†Hepatocellular carcinoma</td>
</tr>
</tbody>
</table>

*Pooled mean for cirrhotic specimens = 3-2 (SD 0.36)
*Pooled mean for cirrhotic specimens = 3-41
†Pooled median for HC specimens = 7.3 (SD 1.64)
†Pooled median for HC specimens = 7.83

AgNOR sites were strongly stained as black nuclear dots of varying sizes. When only one or two AgNORs were present they were usually about 1–2 μm diameter; when multiple sites were stained they were smaller and varied in size, from 0.5 to 1 μm diameter. There was no significant difference between the values for normal livers (mean 1.88 AgNOR/nucleus; median 1.95; SD 0.24; 95% confidence interval 0.78–2.12), adenomas (mean 2.3; median 2.3; SD 0.1; 95% CI 2.21–2.40), and chronic active hepatitis (mean 2.12; median 2.15; SD 0.29; 95% CI 1.94–2.34) (U = 24, overall; p = not significant). This group, however, was significantly different from cirrhotics (mean 3.21; median 3.03; SD 0.33; 95% CI 2.79–3.25) (U = 0; p < 0.001) which in turn were also significantly different from the group.

Fig 1  "Scattergram" of AgNOR numbers in the hepatic specimens examined. There is separation of the values for normality, cirrhosis, and hepatocellular carcinoma. Liver cell "dysplasia" values lie intermediate between cirrhosis and carcinoma.
with hepatocellular carcinoma (mean 7·88; median 6·90; SD 2·9; 95% CI 4·90–8·86) (U = 0; p < 0·001). In the specimens containing established cirrhosis with hepatocellular carcinoma, the counts in the two regions differed greatly (cirrhotic areas: 3·32 mean AgNORs/nucleus; median 3·25; SD 0·39; 95% CI 2·80–3·69; hepatocellular carcinoma areas: 7·46 mean AgNORs/nucleus; median 7·0; SD 1·76; 95% CI 5·1–9·0) (U = 0; p < 0·001). Intermediate values were seen in the four cases of cirrhosis judged to possess liver cell dysplasia (mean 4·48 AgNORs/nucleus; median 4·20; SD 0·99; 95% CI 2·77–5·5) (U = 2; p < 0·05 compared with hepatocellular carcinoma; U = 2; p < 0·05 compared with cirrhosis) (figs 1–4).

In the series of specimens of uncertain histology four had “low” AgNOR counts (range 2·7–3·5/nucleus; mean 3·2; median 3·41; SD 0·36; 95% CI 1·01–7·84) and three had “high” values (range 6·1–9·3/nucleus; mean 7·5; median 7·83; SD 1·64; 95% CI 2·79–12·83). In two of the first four specimens the diagnosis lay between normality and cirrhosis and in the second pair between cirrhosis and hepatocellular carcinoma; but the values were all in the cirrhotic range. On rebiopsy (within two weeks) the diagnosis proved to be cirrhosis in all four specimens. In the remaining three cases the diagnostic uncertainty related to cirrhosis v hepatocellular carcinoma; the range of AgNOR counts was within the hepatocellular carcinoma range in all three and, indeed, this proved to be the correct diagnosis on rebiopsy (and, in two cases, necropsy) in each of the three. As in previous
NORs in liver

NORs in liver

studies reproducibility of counts was 3–5% (intra-
observer and interobserver).

Discussion

NORs have recently found several applications in
diagnostic and research histopathology. These
structures, which may be seen in D and G group
acrocentric chromosomes 13, 14, 15, 21 and 22 in man,
in metaphase, and as fibrillar centres in interphase
nuclei, are loops of ribosomal DNA. Their sizes and
numbers vary with the degree of differentiation in cell
lines in vitro, as shown for example, in human
promyelocytic leukaemic (HL60) cells, where
induction of differentiation leads to diminution of
NOR numbers. Conversely, in phytohaemagglutinin-
in-stimulated “blast transformation of human peri-
pheral blood lymphocytes,” NOR numbers increase.
The reason for this phenomenon is uncertain. It could
be related to increased ploidy, but DNA flow cytometry of non-Hodgkin’s
lymphomas has produced no evidence for this. Another possibility is that NOR numbers in
interphase are part of a fundamental process related to
cell proliferation and transformation; certainly, in
human lymphomas, there is a highly significant
association and correlation between NOR counts and
the numbers of cells labelled with the monoclonal
antibody Ki67 which seems to be a marker of
proliferating cells. Other possible determinants of the
numbers of NORs in a particular cell include rDNA
gene amplification phenomena, the stage of the cell
cycle, and circadian rhythms and age, whose effect is as
yet uncertain.

It has been shown, however, that the enumeration of
silver stained interphase NORs in a variety of human
tissues may provide valuable, often diagnostic, data.
NOR counts distinguish between high and low grade
non-Hodgkin’s lymphomas, malignant melanomas and
melanocytic naevi, skin adnexal tumours, “oat
cells” and lymphocytes, benign and malignant
pleural mesothelial cells, types of small round cell
 tumours and benign and malignant fibroblastic
lesions of childhood, benign and invasive transitional
cell tumours of the nose, and benign and malignant
salivary gland neoplasms. Other tissues, notably
those of endocrine type and some surface epithelia,
yield less useful distinctions between hyperplastic and
benign and malignant neoplastic lesions. The reason
for this is obscure but could, in part, result from
continued stimulation or proliferation of normal
cells, at least in some endocrine organs, with gene
amplification.

In this study we have shown that the simple,
reproducible AgNOR method can be applied to
problems of liver disease. It may often be difficult in
small needle biopsy specimens to distinguish between
normality or cirrhosis (especially of the macronodular
variety), or between cirrhosis and hepatocellular
carcinoma when the latter is exceptionally well
differentiated. The ranges and pooled means of
AgNOR counts for these lesions are widely separated
and would enable the distinction to be made readily,
even in small specimens. The same is true for
adenomas and hepatocellular carcinoma, where
histological distinction may be difficult. The increased
numbers of AgNORs in the cells of cirrhosis
presumably relate to increased cell turnover in
regeneration nodules, but these numbers did not
approach those observed in hepatocellular carcinoma,
where cell proliferation is presumably even more
pronounced.

It is also of interest that in cirrhosis with dysplasia the
AgNOR numbers lay between those in cirrhosis
alone and hepatocellular carcinoma. This may
represent rDNA gene amplification in premalignancy.
In Novikoff hepatoma cells in vitro, an excess of
abnormal NOR complexes has been reported over
normal or regenerating liver cells. Furthermore, the
AgNOR counts in seven biopsy specimens of
equivocal pathology were able to predict the type of
disease, as confirmed by repeat biopsy (or necropsy)
within a short time from the initial biopsy.

These findings underline the usefulness of this
technique in diagnostic histology. A recent brief
review questioned the technical ease of the method and
its reproducibility. There have, in fact, been no
difficulties in attaining intra- and interobserver errors
of only 1–5% in AgNOR enumeration, as recorded in
several recent papers. Furthermore, painstaking
attention to water purity has solved any problems
which have occurred in our laboratories (and in those
seeking our assistance).

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