Dysplastic Nodules and Hepatocarcinogenesis in Cirrhotic Explants

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Introduction

In recent years, a growing literature has supported the concept that large nodules usually found in cirrhotic livers represent premalignant lesions in the setting of chronic liver disease. With the use of advanced imaging techniques¹⁻³, nodules suspicious for malignancy have often been identified and resected. While some resected lesions were found to be small hepatocellular carcinomas (HCCs)³⁻⁵, others were not. Some of these non-malignant nodules were devoid of atypia, some had architectural or cytological atypia insufficient for a diagnosis of HCC though suggestive of a premalignant state, while others contained microscopic subnODULES of HCC³⁻⁷. In follow-up studies⁸⁻¹⁰ and series of explants from liver transplant centers¹¹⁻¹⁵, the occasional finding of microscopic foci of HCC in the nodules was confirmed and significant associations with HCC elsewhere in the same liver were established. Such findings suggested that these nodular lesions are probably a frequent pathway of human hepatocarcinogenesis in a wide array of liver diseases and in diverse populations of patients.

Interestingly, the emphasis in these early lesions has been somewhat different depending on the population of patients being studied. In Korea and Japan, where the bulk of available pathologic material comes from clinical situations resulting in biopsies and resections of lesions, a bit more emphasis has been place on higher grade lesions and early HCCS, these being the most likely to be detected radiologically and the ones most closely associated with clinical progression and therefore of the greatest import¹⁵, ¹⁶. On the other hand, western countries where large scale screening of patients at risk is not routine, most of the lesions have been identified and studied in the transplant setting, where a whole cirrhotic liver is available for examination. This source of pathologic material for study has the advantage of being able to survey even those lesions that might not be detectable by conventional imaging, but, on the other hand, fails to yield any follow-up data regarding the behavior of the lesions. Thus, all
conclusions are based on associations, rather than observed progression.

**Definitions and Observations**

Dysplastic nodules consist of hepatocytes and almost always contain some intact, normal appearing, portal tracts. Since they are most often found in the setting of cirrhosis they seem to correspond to what Edmondson called "adenomatous hyperplasia" (AH)\(^9\), though he viewed these lesions as having "limited growth potential". Other terms used include macroregenerative nodule (MRN)\(^8,11-13\), dysplastic nodule\(^6\), hepatocellular pseudotumor\(^14\), and adenomatoid hyperplasia\(^15\). AH has been most widely used, though not exclusively, by researchers from Japan. MRN, coined by Furuya et al for the first Japanese autopsy study of the nodules\(^8\), became the most widely accepted term in the earliest publications from North America and Europe. In 1997 a consensus document co-authored by world wide investigators rejected all of these terms as being imprecise if not actually misleading, suggesting the term "dysplastic nodule", with further classification as low or high grade, to replace them\(^30\). In this review we will use dysplastic nodule as the preferred term, however, recognizing at the same time that it, too, is problematic.

DNs are defined grossly as large hepatic nodules that are distinct from the surrounding liver parenchyma in terms of size, color, texture, or the degree to which they bulge from the cut surface of the liver (Fig 1). Confirmation that a nodule is in fact a DN comes with histologic examination and the identification of intact portal structures distributed through the lesion. The number of these portal structures may be reduced compared to a similar area of non-diseased hepatic parenchyma, but may also be virtually normal in number and distribution. In a lesion consisting of well-differentiated hepatocytes, the presence of portal structures confirms that the lesion is not actually hepatocellular carcinoma (or adenoma, in a non-cirrhotic liver).

![Figure 1. Sections of a liver with hepatitis B cirrhosis. The largest nodule at the lower right was histologically shown to be hepatocellular carcinoma. The other distinctive nodule, at the upper left edge of the specimen, was histologically demonstrated to be a high grade dysplastic nodules with sub-nodules of hepatocellular carcinoma.](image-url)
Table 1. Features which may be found in Low grade (ordinary) and High grade (atypical) DNs.

<table>
<thead>
<tr>
<th>Low grade DNs</th>
<th>High grade DNs</th>
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<tr>
<td>Normal architecture</td>
<td>Focal Pseudogland formation</td>
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<tr>
<td>Normal cytology or large cell change</td>
<td>Diffuse small cell change</td>
</tr>
<tr>
<td>Iron accumulation in otherwise</td>
<td>Nodule-in-nodule lesions with:</td>
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<tr>
<td>non-siderotic liver</td>
<td>Small cell change</td>
</tr>
<tr>
<td>Iron resistance in otherwise</td>
<td>Iron accumulation</td>
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<tr>
<td>siderotic liver</td>
<td>Iron resistance in siderotic nodule</td>
</tr>
<tr>
<td>Diffuse fatty change</td>
<td>Fatty change</td>
</tr>
<tr>
<td>Copper accumulation</td>
<td>Clear cell change</td>
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<tr>
<td>Schirrous growth in the absence of other atypia</td>
<td>Mallory body clustering</td>
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<td></td>
<td>Decreased reticulin fibers</td>
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DNs have been found in a wide variety of chronic liver diseases\textsuperscript{11–13}, including processes which are hepatitic (hepatitis B, C, and autoimmune hepatitis), cholangitic (primary biliary cirrhosis, primary sclerosing cholangitis), metabolic (alpha-1-antitrypsin deficiency, primary hemochromatosis), and toxic (alcoholic liver injury). Typically, livers with DNs contain a small number of these nodules, rarely more than ten, although there are exceptions which will be discussed further on\textsuperscript{8–13}. DNs may be sub-classified as low grade or high grade according to features listed in Table 1. Low grade lesions may be devoid of atypia or display features of large cell change. They may be iron or copper retentive or they may be diffusely steatotic. They should not contain nodule-in –nodule type lesions or small cell change, let alone features closely associated with HCC -- these changes would be indicative of the lesion being high grade. It is usually not possible at this time to radiographically distinguish small HCCs from DNs or high grade DNs from low grade DNs with complete confidence\textsuperscript{21, 22}, nor is it usually possible to reliably make such distinctions on the basis of gross morphology. Histologic examination, either by biopsy or examination of a resected specimen, is required for accurate classification.

1. Histologic Features of Low grade DNs

Low grade DNs are very well defined nodules, being surrounded by a condensed rim of fibrous tissue similar to that surrounding cirrhotic nodules (Fig. 2). The nodules are thus not truly encapsulated. Portal tracts, present in virtually all DNS, are most often uniformly distributed in low grade DNs and may even be distributed in a virtually normal fashion with regularly intervening terminal hepatic venules. In some nodules, portal structures may be caught up in fibrous septa which partially subdivide the nodule.

The hepatocytes of low grade DNs tend to be of comparable size to hepatocytes outside the lesion. The hepatocytes may display changes characteristic of the underlying liver disease affecting the surrounding
liver, such as fatty change, Mallory bodies, or increased iron or copper deposition. These changes will be distributed in the DN as they are in surrounding cirrhotic nodules. Occasionally, a DN in a non–siderotic liver may contain increased iron or a DN in an otherwise siderotic liver will be iron free; it would still be classified as low grade. Rarely, a similar increase in copper, in an otherwise copper–free liver, is also seen. These changes in DNs, when not confined to a subnodule within the lesion, i.e. copper or iron retention and fat accumulation, may represent a marker of the clonality in the DN hepatocytes. We will return to this subject below.

2. Histologic Features of High grade DNs

High grade DNs are defined by the presence of small cell change and/or architectural atypia (Fig. 2). They are usually well circumscribed and surrounded by a condensed rim of fibrous tissue, like low grade lesions, though some may merge focally with adjacent liver parenchyma.23 The atypical features in high

Figure 2. Histological features of (A) low grade dysplastic nodule, (B) high grade dysplastic nodule with small liver cell dysplasia (arrows), (C) dysplastic nodule with hepatocellular carcinoma foci (arrows), (D) hepatocellular carcinoma with trabecular pattern (H–E, x100).
grade DNs may take a variety of forms and may be diffuse throughout the nodule or focal. Diffuse changes most often fall into the category of cellular atypia. The definition of cellular atypia in this setting should be limited to small cell change: small, crowded hepatocytes with basophilic cytoplasm and an increased nuclear–cytoplasmic ratio \(^{24}\) -- appears to be consistently related to the development of HCC in a variety of studies and should remain a criterion for "atypical." Pseudoacinar structures resembling those seen in well differentiated HCC are a form of architectural atypia in high grade DNs and may be either focal or diffuse.

Focal atypia may merge with the surrounding DN parenchyma, but it more often occurs instead as a "nodule-in-nodule" lesion \(^{13}\). Such subnodules often appear to compress the adjacent DN parenchyma and studies of proliferative rates of the cells making up these lesions indicate that they are proliferating more rapidly than the surrounding tissue \(^{25}\). These subnodules may display small cell change \(^{11, 24}\), but may also show changes which are not classically "atypical" including fatty change \(^{26}\), clear cell change \(^{11, 22}\), clusters of hepatocytes with Mallory’s hyaline \(^{27}\), increased iron uptake within the DN \(^{28}\), iron resistance in an otherwise siderotic nodule \(^{28, 29}\), and accumulation of copper binding protein \(^{30}\). Some expansile subnodules do not display any distinctive cytological features though, architecturally, they may display a pseudoacinar growth pattern. We have argued that all subnodules, with or without distinctive cellular changes, are appropriately defined as architectural atypia on the basis of the expansile growth and should warrant classification of the entire DN as a high grade lesion \(^{13}\).

HCC may be identified in high grade DNs (Fig. 2)\(^{3, 5, 6, 8-14, 30-33}\). These microfoci of HCC may display any of the features seen in larger HCCs, though they are usually well–differentiated. Typical growth patterns include pseudoacinus formation, thickened trabeculae or a schirrous growth. Common cytological features include intracytoplasmic Mallory’s hyaline, fatty change, clear cell change, iron resistance, and multinucleation. Multiple foci of HCC may also be found in a single DN and the histologic features of these foci are often different from each other \(^{31, 33}\). The DN parenchyma surrounding a microfocus of HCC will usually contain portal tracts and may consist of normal appearing hepatocytes, suggesting a background of a low grade MRN, or may show atypia, indicating a high grade background. Either way, by convention, DNs containing foci of HCC are classified as high grade.

**The Premalignant Nature Of DNs**

The association of DNs with HCC is demonstrated in two ways. First, as mentioned above, DNs sometimes contain one or multiple microscopic foci of HCC. Second, DNs are sometimes found in livers which also contain grossly apparent HCC elsewhere. Both of these relationships have been found to achieve statistical significance \(^{11-13}\). Beyond this statistical correlation, the atypical features often found in
DNs include many which have previously been thought to be premalignant. Clustering of hepatocytes containing Mallory’s hyaline\textsuperscript{34,35} and foci of iron resistance in siderotic nodules\textsuperscript{39,40} have been independently described as premalignant changes. Small liver cell dysplasia (LCD), considered premalignant on the basis of morphometric analysis, is also seen and, in some series, is very common\textsuperscript{5,10-14,30}.

Other features which are commonly identified in mature HCC are found in high grade DNs. For example, immunohistochemical studies of DN sinusoids reveals increasing degrees of “capillarization” (i.e. loss of endothelial fenestration, deposition of basement membrane and expression of antigens such as factor VIII and CD34) with the development of atypia and HCC (Fig. 3\textsuperscript{37,38}). Expression of vascular endothelial growth factor also increased in high grade DN and HCC\textsuperscript{39}. Studies of ploidy indicate increased frequency of aneuploidy in high grade DNs\textsuperscript{40}. Immunohistochemical staining for AFI\textsuperscript{41} demonstrates expression of these proteins in atypical foci and microfoci of HCC. Reactivation of telomerase activity was found with the development of atypia and HCC\textsuperscript{42,43}. Clonal stepwise progression

\textbf{Figure 3.} (A) Sinusoidal capillarization demonstrated by immunohistochemical staining for CD34 in low grade DNs, (B) high grade DNs, (C) DNs with HCC foci in the right side, and (D) HCCs. Notice that clearly increased sinusoidal capillarization with the progression of human multistep hepatocarcinogenesis (LSAB, x100).


has also been demonstrated in a subnodule of HCC arising in a DN by Tsuda et al.\(^{40}\). In their study, they exploited integration of the hepatitis B surface antigen gene into host hepatocytes using restriction length polymorphism analysis, demonstrating that a microfocus of HCC consisted of cells derived from the same clone as the surrounding DN parenchyma. We will return to the issue of clonality below.

These findings all represent static samplings of the process of malignant transformation. From the association of DNs with HCC elsewhere in the same liver we deduce that DNs are at least a marker of a liver with a tendency to generate malignancies. Several studies however now demonstrate that, when followed over time by serial biopsies or biopsy and then radiographic changes indicative of expansile growth, there is in fact progression from at least high grade DN to HCC\(^{45-49}\).

In these longitudinal studies, it is interesting to note that low grade DNs are not as obviously premalignant or as indicative of neighboring malignant transformation as are high grade DNs. The data addressing this difference appears contradictory. Studies of the various aspects of DNs mentioned — proliferation\(^{14, 40-51}\), p-ras expression\(^{14}\), ploidy\(^{40}\), endothelial and extracellular matrix alterations\(^{37, 38}\), telomerase activity\(^{42, 43}\) — find similarities between high grade DNs and HCC. In the absence of these similarities between low grade DNs and HCC, most of these researchers conclude that high grade lesions are related to HCC, whereas low grade lesions are related to regenerative nodules and are less clearly implicated in hepatocarcinogenesis.

Data from the largest series of liver explants both supports and goes against this view\(^{13}\). On the one hand, a subset of livers was identified (n=13, of 155) which contained so many DNs as to be virtually uncountable. When large LCD was excluded as a criterion for atypia, in all but one of these livers all the sampled DNs were low grade. None of these livers contained HCC. All such livers were in patients with chronic hepatitis (hepatitis B, hepatitis C, or autoimmune hepatitis) and the mean age of these patients was significantly lower than the other patients with few or no DNs. These findings suggest that these DNs are actually large regenerative nodules that have not yet scarred down into smaller nodules, examples of the original “adenomatous hyperplasia” of Edmondson rather than neoplastic lesions. They might also correlate with large cirrhotic nodules which became undetectable on long term ultrasound surveillance in a study by Kondo et al\(^{72}\). On the other hand, statistical associations of low grade lesions with co-existent HCC in this, as in earlier, series are strong even when high grade lesions are excluded from analysis\(^{11, 13}\). Thus, the neoplastic, pre-malignant nature of low grade lesions seems more open to question than it does for high grade lesions. An explanation for these discrepancies which possibly resolves the apparent contradictions has been suggested on the basis of animal models of hepatic progenitor stem cells\(^{53}\).
Speculations on the Early Stages of Human Hepatocarcinogenesis

How DNs actually form has not yet been firmly established. One early view suggested that an ordinary regenerative nodule in cirrhosis becomes more rapidly proliferative, therefore becoming larger. In turn, with the increased proliferation it also becomes at greater risk for the carcinogenic "hits", thereby giving rise to atypia and carcinoma\(^7\). While simple, this hypothesis does not take into consideration three known facts about DNS. First, they can be found in livers in advance of cirrhosis and therefore do not always arise from a pre-existent regenerative nodule\(^8,33,54\). Second, the presence of many intact portal tracts in most DNs, which have not yet been demonstrated to fully reconstitute after scarring and injury, suggests that they must be pre-existent to the formation of the DN, making it unlikely that a small cirrhotic nodule with few if any portal tracts could enlarge to a nodule with many portal tracts. Third, some DNs have been demonstrated to be clonal lesions, not hyperplastic phenomenon\(^44,55-57\).

To account for these features, we have suggested an alternative process of DN development\(^33,33,98\). This alternate hypothesis has been able to predict some previously unexplored features of DNs and, perhaps, can be broadened to explain other types of borderline or early carcinoma features. The steps of this hypothesis are as follows:

i) A clonal expansion of hepatocytes follows on the earliest carcinogenic events in response to any diffuse injury of the liver which leads to increased hepatocyte turn over;

ii) These early hits lead to a clonal expansion of hepatocytes which spreads around adjacent portal structures rather than displacing them;

iii) As the rest of the liver becomes scarred, progressing to later stages of disease and eventually cirrhosis, the island of clonal hepatocytes, if resistant to the scarring affecting the rest of the liver, would remain intact -- an island of relatively preserved hepatic parenchyma made up of neoplastic, clonal hepatocytes;

iv) With establishment of cirrhosis in the adjacent liver, the clonal expansion takes on the appearance of a large cirrhotic nodule;

v) Having already undergone the earliest transforming events of hepatocarcinogenesis, the clonal, hepatocyte expansion remains at increased risk for later developments and, thus, the lesion becomes the likeliest site of full malignant transformation.

1. Proliferation Rates of DNs

On the basis of this alternate hypothesis we made a testable prediction about proliferation rates in DNs. This prediction was based on the idea that the spreading clonal expansion preceded the development of cirrhosis and therefore could take perhaps years to develop. Thus, it was possible that
dysplastic nodules could have relatively low proliferative rates, rather than the high proliferative rates necessitated by the earlier model. In fact, this prediction was confirmed: low grade DNs and the background parenchyma of high grade DNs, outside of atypical subnodules, had proliferation rates that were similar or lower than that of surrounding cirrhotic nodules. Careful analysis of other studies of proliferation \(^{14, 49-51}\) demonstrate the same thing, though most investigators do not separate atypical foci from background non-atypical hepatocytes in high grade DNs in performing their analyses.

It is this latter mode of analysis that suggests that high grade DNs are rapidly proliferative lesions when, in fact, it is the atypical foci that have increased proliferative rates, while the background DN is not usually elevated. Thus most authors in most DN proliferation studies merely join high grade DNs together with HCC as high proliferative lesions, obscuring the transition from low grade DN (low proliferative) to atypical foci (high proliferation) arising in a low proliferative background in high grade DNs, to HCC (very high proliferation).

2. Apoptosis in DNs

If dysplastic nodules have relatively low proliferative rates we must find an alternate explanation for the ability of these hepatocytes to displace surrounding hepatic parenchyma. Of course, even if not rapidly proliferative, if these hepatocytes had a survival advantage compared to surrounding non-neoplastic hepatocytes they would expand in a clustered fashion. One possibility is that they might be resistant to the disease damaging the surrounding parenchyma (e.g. inability to be virally infected, absence of antigen presentation once infected, etc.) or they might have impaired mechanisms of apoptosis.

We tested this latter possibility using by performing terminal deoxynucleotidyl transferase–mediated dUTP–biotin nick end labeling (TUNEL) assay and proliferation cell nuclear antigen (PCNA) staining in low grade DNs, high grade DNs, small HCCs, and surrounding cirrhotic nodules. The PCNA data confirmed the earlier studies did not have increased proliferative rates over surrounding regenerative nodules, and increases in proliferation only occurring with the development of atypia. Apoptosis did appear diminished in low grade DNs, only increasing with the emergence of atypia. The rates of apoptosis in low grade DNs were not significantly lower than those of surrounding regenerative nodules, however the ratio of apoptosis to proliferation was increased in regenerative nodules, indicating that the hepatocyte populations, as expected, did have a survival advantage when compared to non-DN hepatocytes.

3. Hepatic Stellate Cell (HSC) Activation in DNs

The key feature of our hypothesis of DN development is that the nodular appearance arises not from expansile growth, but because the clonal hepatocytic expansion with reduced or absent scarring while the
surrounding, non-neoplastic liver becomes cirrhotic. Thus we made a second testable prediction: that there would be diminished stellate cell activation in dysplastic nodules. Again, our prediction was confirmed\(^{(50)}\): activated stellate cells, as demonstrated by immunohistochemical staining for smooth muscle actin, were in significantly few number within dysplastic nodules than in the surrounding cirrhotic nodules. To our knowledge no other studies of this aspect of DN pathophysiology have been reported.

4. Spreading of Clonal Expansions

The clonality of many, if not all, dysplastic nodules has been demonstrated in a number of studies. Interestingly, many regenerative–appearing nodules have also been identified as clonal expansions, and still more intriguingly, neighboring, clustered smaller cirrhotic nodules are often of the same clone\(^{(50)}\). This suggests, once again, that the clonal expansion preceded the development of cirrhosis and that scarring subsequently subdivided the clonal expansion into separate nodules. Moreover, clonality of fields of hepatocytes has been identified in advance of cirrhosis, even in the absence of liver disease\(^{(50)}\). This suggests that some clonality is not related to neoplastic transformation but is rather a product of liver tissue formation in embryogenesis. However, these data confirm that clonal expansions can in fact occur without displacing original portal structures, as we predicted in our model of DN development. How this precedes architecturally remains unclear. But in animal models of hepatocyte transplantation in which a small population of transplanted cells completely repopulates a genetically damaged liver, such as in the tyrosinemia mice of Markus Grompe and colleagues \(^{(61)}\), complete repopulation around native structures, is readily demonstrated.

Summary

In the last decade, careful examination of cirrhotic livers has confirmed that dysplastic nodules represent hepatic premalignant lesions in the setting of chronic liver disease. Careful examination of their gross and microscopic morphologies has led to our suggested hypothesis of pre–cirrhotic, spreading clonal expansions, resistant to scarring, which result in neoplastic islands of hepatic parenchyma. The resultant distinctive nodules, often marked by features suggestive of their clonality (such as increased pigment), are at increased risk for subsequent carcinomatous events, and thereby give rise to HCC. Specialized molecular and immunohistochemical studies confirm many aspects of this hypothesis.

Key Words: Dysplastic nodule, Hepatocellular carcinoma
References


