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Vascular architecture: is it a helpful histopathological biomarker for hepatocellular carcinoma?

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Abstract: Hepatocellular carcinoma (HCC) remains one of the major public health problems throughout the world. Although originally associated with tumorigenic processes, liver angiogenesis has also been observed in the context of different liver inflammatory, fibrotic, and ischemic conditions. Here we investigate the fractal dimension as a quantitator of non-Euclidean two-dimensional vascular geometry in a series of paired specimens of primary HCC and surrounding non-tumoral tissue, and discuss why this parameter might provide additional information regarding cancer behavior. The application of fractal geometry to the measurement of liver vascularity and the availability of a computer-aided quantitative method can eliminate errors in visual interpretation, and make it possible to obtain closer-to-reality numerals that are compulsory for any measurement process.

Key words: Liver, Angiogenesis, Cancer, Fractals, Geometry, Biomarkers
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Hepatocellular carcinoma (HCC) represents the fifth most common cancer worldwide (Zerbini *et al*., 2006; Jemal *et al*., 2005); it is the most common primary cancer associated with hepatitis B and hepatitis C virus chronic infections, alcohol abuse, or in developing countries with food contaminated with *Aspergillus flavus* fungus. A variety of genomic and molecular alterations have been identified in fully developed HCC and to a lesser extent in morphologically defined precancerous lesions (Thorgeirsson *et al*., 2006; Farazi and DePinho, 2006). HCC is a complex non-linear disease that emerges from multiple spontaneous and/or inherited mutations that induce dramatic changes in expression patterns of genes and proteins that function in networks controlling critical cellular events. Therefore, it is compulsory to identify HCC and the recurrence at its earlier period. A number of serum markers have been proposed and currently used for detecting HCC, none provides a real quantitative index for predicting patient outcome (Zhou *et al*., 2006; Grizzi *et al*., 2007).

Angiogenesis refers to the dynamic process of generating new vessels as a result of the sprouting and branching of capillaries from pre-existing arteries and veins (Carmeliet, 2003). Although associated with tumorigenic processes, angiogenesis has also been observed in the context of different liver inflammatory, fibrotic, and ischemic conditions (Medina *et al*., 2004). Despite substantial progress has been made in various aspects of angiogenesis, there is still no univocal consensus concerning the most useful method of quantifying vasculature in two-dimensional histological sections (Sharma *et al*., 2005). One crucial difficulty is that the vascular system is irregularly shaped. This not only makes it difficult for even expert morphologists to provide an objective estimate, but also hampers the identification of numerals capable of representing the close-to-reality variables that describe the vascular architecture observed through a light-microscope (Grizzi *et al*., 2001). The most used histopathological index of tumoral vasculature is micro-vessel density (MVD) (Sharma *et al*.,

2005). However, MVD has a number of substantial limitations, which are mainly due to the biology of tumour vasculature (Hlatky *et al*., 2002) and the non-Euclidean geometry that the vascular system assumes in real space (Grizzi *et al*., 2001). The latter cannot be measured using the principles of Euclidean geometry, because this can only interpret regular objects that are almost impossible to find in nature (Mandelbrot, 1983).

The main histomorphological feature of vasculature is the diversity of the sizes, shapes and connecting patterns of the vessels, which inevitably gives rise to highly variable numbers when considered in two-dimensional histological sections (Marion-Audibert *et al*., 2003; Grizzi *et al*., 2001). All of these features are the main causes of the errors in visual interpretation and discordant results concerning different laboratories' assessments of the same tumour. However, the vascular system can be geometrically designed as a fractal network of irregularly branching vessels whose lengths and diameters became systematically smaller. The vascular system fulfils four of the main properties of natural fractal objects: (1) it has an irregular shape; (2) it has a non-integer or fractal dimension (*D*); (3) it demonstrates statistical self-similarity, which means that its parts statistically resemble the whole; and (4) it is subject to scaling, which means that its measured properties depend on the scale at which they are observed.

One fundamental concept for evaluating geometric spaces is that of dimension, which has mainly been defined in two ways (Grizzi *et al*., 2001). The first or "topological" dimension assigns an integer number to every point in Euclidean space and attributes 0 to a point (defined as that which has no part), 1 to a straight line (defined as a length without width), 2 to a plane surface (defined by its length and width), and 3 to a three-dimensional figure (defined by its length, width and depth). The second definition attributed a real number to every natural object in E3 lying between the topological dimension and 3. The dimension of a two-dimensional section of the vascular system falls between 0 and 2. The more *D* tends towards 2, the more the analyzed vascular configuration tends to fill a space and the greater its geometrical complexity.

Here we investigate the fractal dimension as a quantitator of non-Euclidean two-dimensional vascular geometry in a series of paired specimens of primary HCC and surrounding non-tumoral tissue, and discuss why this parameter might provide additional information regarding cancer behavior.

Two-micrometer thick liver sections taken from patients (n=8, mean age=71, range from 61 to 82 years old, male:female=6:2) with primary HCC were immunohistochemically treated with antibodies against CD34 (Dako, Milan, Italy) in order to visualize their vascularity. For each histological section, two >10 mm^2 areas separated by a distance of 5 mm were identified: one representing tumoral tissue and the other a portion of non-tumoral and non-cirrhotic surrounding parenchyma. All of the areas were automatically digitised using a computer-aided image analysis system consisting of a Leica DMLA microscope (Leica, Italy) equipped with an x–y translator table, digital camera (QImaging, UK), and a computer with incorporated ad hoc constructed image analysis software that automatically selected the immunopositive vessels on the basis of RGB color segmentation. All of the measurements were made at 20× objective magnification. The vascular surface was automatically quantified as the sum of the areas of the CD34-immunopositive vessels, and was expressed as a percentage of the liver biopsy section surface area. The vascular fractal dimension was automatically estimated using the box-counting method which applies the equation:

$$D=\lim_{\varepsilon\to 0}\frac{\log N(\varepsilon)}{\log(1/\varepsilon)}, \tag{1}$$

where D is the box-counting fractal dimension of the immunoreactive vascular surface, ε the side length of the box, and $N(\varepsilon)$ the smallest number of contiguous and non-overlapping boxes of side ε required to cover the immunoreactive vascular surface completely. As the 0 limit cannot be applied to biological images, D was estimated by means of the equation:

$$D=d, \tag{2}$$

where d is the slope of the graph of $\log N(\varepsilon)$ against $\log(1/\varepsilon)$. As natural objects are scale invariant, they maintain their fractal dimension in a fixed range of side lengths (ε_{min}–ε_{max}) based on the constant fitting

parameter *D*.

The minimum and maximum values of vascular surface obtained by measuring non-tumoral vascularity were 0.7% and 3.41%, with a mean value of (1.84±1.09)%, and those obtained by measuring tumoral vascularity were 0.78% and 7.47%, with a mean value of (4.46±2.11)% (Fig.1a). Analysis of variability showed *CV* (coefficient of variability)=59% for the non-tumoral vascular surface, and *CV*=47% for the tumoral vascular surface. Spearman's correlation between the paired non-tumoral and tumoral vascular surface fractal dimensions was r=0.62 (P<0.01).The minimum and maximum values of *D* obtained by measuring non-tumoral vascularity were 1.36 and 1.60, with a mean value of 1.47±0.09, and those obtained by measuring tumoral vascularity were 1.38 and 1.72, with a mean value of 1.62±0.11 (Fig.1b). Analysis of variability showed *CV*=6% for the non-tumoral vascular surface fractal dimensions, and *CV*=7% for the tumoral vascular surface fractal dimensions. Spearman's correlation between the paired non-tumoral and tumoral vascular surface fractal dimensions was r=0.39 (P<0.01).

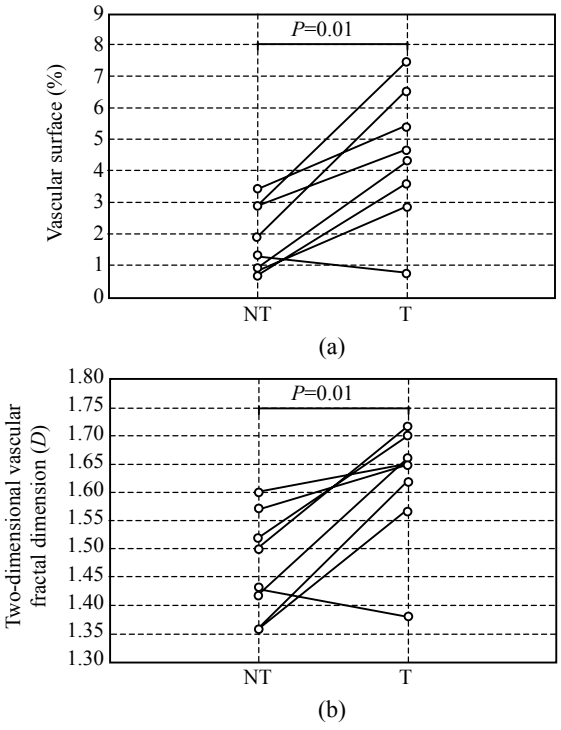

Fig.1 The vascular surface (a) and fractal dimension (b) were automatically estimated in 8 paired non-tumoral (NT) and tumoral (T) tissues

The non-linear, temporal and spatial advancement of the promotion, progression, mediation and inhibition states of angiogenesis generates a complex ramified structure that irregularly fills the surrounding environment.

We have recently pointed out that the fractal geometry of tumour vasculature and its well-known biological properties mean that it cannot be measured on the basis of MVD estimates alone (Grizzi *et al*., 2001). The potentially broad applicability of quantitative fractal indices makes it possible to explore the range of the morphological variability of the liver vasculatures, thus increasing the diagnostic importance of such variability in cancer research (Grizzi *et al*., 2001; Grizzi and Chiriva-Internati, 2006). It can be said that:

(1) Liver angiogenesis is a dynamic process whose large number of molecular players make it complex in time and space.

(2) Analysis of the angiogenic process allows the identification of a number of different configuration patterns during a certain time interval, and transitions between two successive states.

(3) This preliminary study suggests *D* as a quantitator of the two-dimensional non-Euclidean geometry of the liver vascular system under normal and neoplastic conditions.

(4) *D* is a quantitative index which depends on the vessel density, shape, size and distribution pattern. The more *D* tends towards 2, the more the analyzed vascular configuration tends to fill a two-dimensional space and the greater its geometrical complexity.

(5) Our findings, whether confirmed in a most large number of cases, stimulate further studies aimed at testing *D* as a quantitator of anti-angiogenic treatments or as a potential means of stratifying patients in clinical trials.

The application of fractal geometry to the quantification of liver neovascularity may be more suitable to its non-Euclidean nature because irregularly tortuous contours and branching structures, such as those seen in tumoral micro-vessels, can be quantitatively characterized by their fractal dimension. The availability of an objective computer-aided method of measurement based on the principles of fractal geometry excludes errors in visual interpretation, and its appropriateness makes it possible to obtain the closer-to-reality scalars that are essential for any

measurement process (Hand, 2004). Furthermore, in the light of recent theories concerning the transient normalization of tumour vasculature (Jain, 2005), fractal geometry can be considered appropriate for quantifying changes in its structure and function, thus leading to the optimization of combined anti-angiogenic and cytotoxic therapies.

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