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RESEARCH ARTICLE

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Bonghan Ducts as Possible Pathways for Cancer Metastasis

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Abstract
Objective: The present study has been designed to find a possible new route for the metastasis of cancer cells on the fascia surrounding tumor tissue using a novel technique of trypan blue staining.
Materials and Methods: Tumor tissues were grown in the skin of nude mice after subcutaneous inoculation with human lung cancer cells. Trypan blue was recently identified as a dye with specificity for Bonghan ducts (BHDs) and not other tissues, such as blood or lymph vessels or nerves.
Results: We demonstrate that the trypan blue staining technique allows the first visualization of BHDs which are connected to tumor tissues.
Conclusion: Since BHDs are known to make up a circulatory system corresponding to acupuncture meridians or collaterals, we propose that, in addition to the currently known blood or lymph vessels, BHDs on tumor tissue fascia may be a novel pathway for metastasis.

1. Introduction

Metastases create major clinical problems in handling cancer patients because treatments effective against the disease confined to the original site are often ineffective against metastatic cancer. Metastasis is currently known to occur via blood vessels, lymphatics, or movement within body cavities and is efficient, in the sense that most human cancers successfully metastasize. On the other hand, it is inefficient as most cancer cells are destroyed in transit. For example, if melanoma cells are injected into an animal's bloodstream, over 99% are destroyed within 24 hours. Another indication of this inefficiency is that about one million cells a day are shed from a mammary cancer, while less than 0.1% of these can be detected in the blood, as they are rapidly destroyed by the host's defense mechanisms [1].

A satisfactory solution has yet to be offered regarding conflicting opinions of the efficiency of metastasis. The question can be posed regarding the possible existence of other unknown, more efficient routes of metastasis. If there is such a route, it may not only resolve the efficiency conflict but also provide new insight into the phenomena that, after an apparently successful treatment of a primary tumor,

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including extended lymphadenectomy, metastases can sometimes arise after an extended period.

In this article, we report the finding of a possible novel path for metastatic cancer. A novel circulatory system, the so-called Bonghan system with Bonghan ducts (BHDs), has been described on the fascia surrounding cancer tissue using an *in situ* trypan blue staining technique. Cancer tissues were grown in the skin of nude mice by subcutaneous injection of human lung cancer cells.

BHDs are anatomical structures corresponding to acupuncture meridians and collaterals and were first found in rabbits and other animals by Bonghan Kim in 1963 [2,3]. However, his results were rarely reproduced [4] and his theory given little credence and ignored for a long time [5,6].

Since the first rediscovery of intravascular BHDs inside the blood vessels of rabbits and rats [7,8], the presence of BHDs has been successively confirmed in various organs, such as lymphatic vessels [9–12], brain ventricles, central canals in the spine [13], and on internal organ surfaces [14]. The entire circulatory network has not been investigated, but evidence of liquid flow in BHD has been produced.

Multiple ductules in a BHD were observed in morphological studies by using hematoxylin and eosin (H&E) staining methods [15] and various types of electron microscopy [16]. Endothelial cells comprising the inner boundaries of the ductules in BHD were different from those in blood or lymph vessels [15]. The liquid flow speeds in BHDs on internal organ surfaces were measured at 0.3 ± 0.1 mm/s [17], with the liquid flow from the skin toward the internal organs observed by injecting chrome-hematoxylin and fluorescent nanoparticles in the skin at a rat testis [18]. An electrophysiological study of a Bonghan system showed a vascular smooth muscle-like excitability [19] and muscarinic receptors [20] that support the circulatory functioning of the Bonghan system.

In this article, we present the trypan blue staining technique recently developed to visualize BHDs [21], without which it would be extremely difficult to visualize their presence on the transparent fascia enwrapping tumor tissue. A histological analysis of a BHD with H&E showed sinuses and flowing cells inside sinuses. Thus the Bonghan circulatory system was found to be connected to tumor tissues, which suggests a possible new route for metastasis.

2. Materials and Methods

2.1. Culture of human lung cancer cells

The cell line NCI-H460 was provided by the Korean Cell Line Bank. Human lung cancer cells were subcultured in a RPMI-1640 medium supplemented with 1% penicillin-streptomycin and 10% fetal bovine serum (FBS) purchased from Invitrogen. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2.

2.2. Animals and cancer model

Female athymic nude mice (aged 5–7 weeks old, weighing 15–20 g, $n=10$; Charles River Laboratories) were used in accordance with institutional guidelines under approved protocols. For subcutaneous xenografts of human cancer, animals were anesthetized with intraperitoneal (i.p.) Zoletil/Rompun and subcutaneously inoculated with 1×10^7 cells (in 1 mL RPMI-1640 medium).

2.3. *In vivo* visualization of Bonghan ducts

Two to 8 weeks after inoculation, the mice were anesthetized with Zoletil/Rompun i.p. and all surgical procedures were performed under general anesthesia. The lateral sides of the tumor skin were incised and the skin over the tumor removed carefully to expose the tumor with an intact outer membrane. A 0.1% trypan blue solution, previously filtered through 0.22-μm pore-sized filter paper, was applied dropwise on the exposed membrane. After rinsing away the dye with warm saline, the identification of Bonghan corpuscles and ducts was assessed using direct visualization with a surgical microscope (SZX12, Olympus).

2.4. Histological analysis

Bonghan ducts and corpuscles were fixed in 10% neutral buffered formalin and embedded in paraffin using routine procedures. Transverse sections of 7 μm thickness were cut with a microtome, stained with H&E, and the samples observed and photographed under a light microscope (BX51, Olympus).

3. Results

With simple microscopy of tumor tissue grown under the skin of a nude mouse, we could not observe any novel, threadlike structures on the surface of the solid tumor after sectioning (Figure 1A). However, with trypan blue staining, BHDs and Bonghan corpuscles (BHCs) were revealed as stained blue. It is remarkable that no other tissues, such as blood vessels or fascia, were stained, but BHDs and BHCs were prominently stained (Figure 1B, C). The full length of BHDs was sometimes untraceable because they entered nearby fatty tissues (Figure 1C). Another notable observation was the detection of a BHD along a blood vessel or nerve bundle connecting the tumor

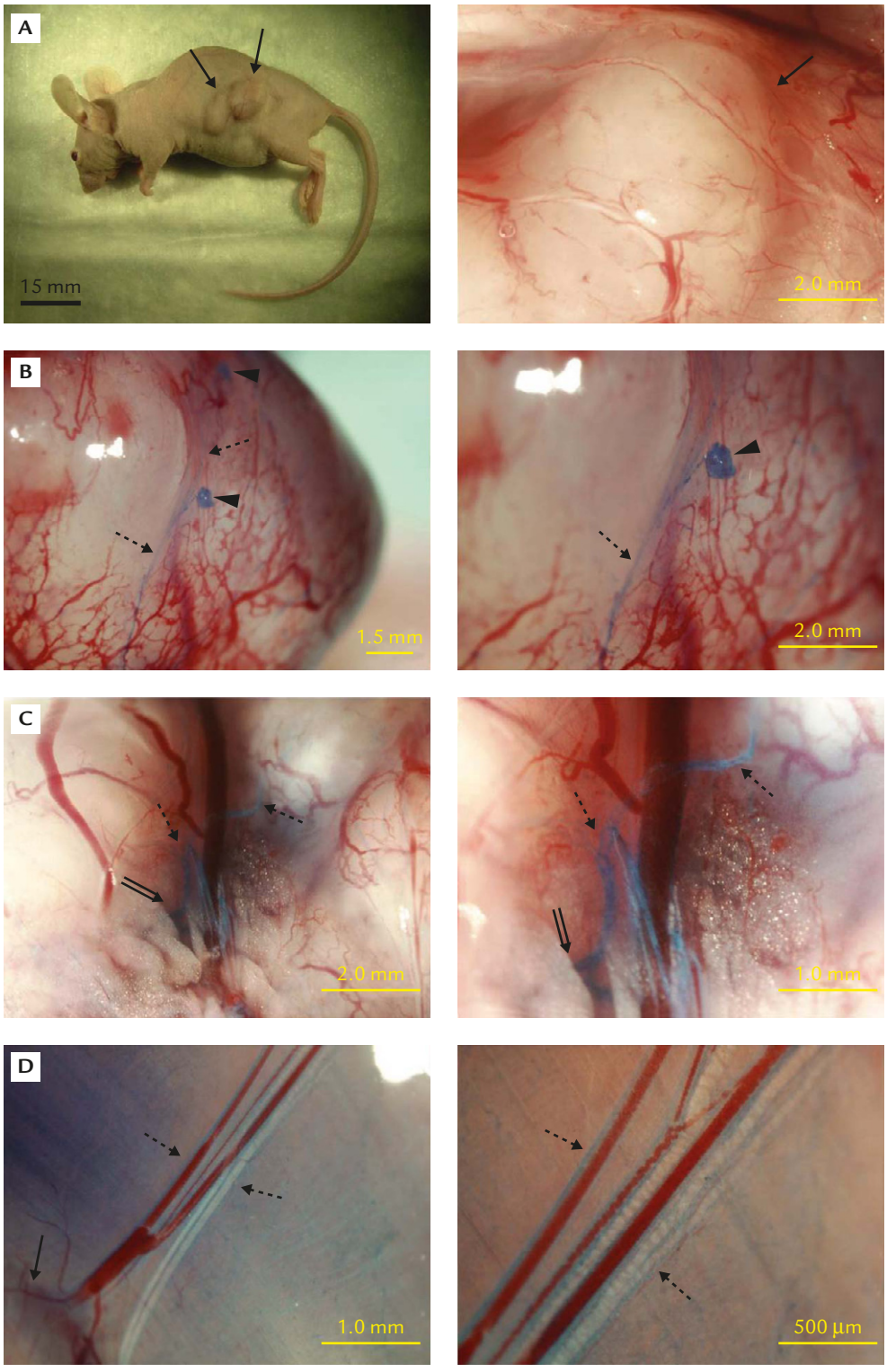

Figure 1 Visualization of the Bonghan system on the fascia surrounding tumor tissue in the skin of a mouse. (A) Images of tumor tissue, indicated by arrows; left: a mouse with two tumors, grown for two weeks after subcutaneous inoculation; right: tumor tissue surface after skin sectioning; Bonghan system hardly detectable. (B) *In situ* trypan blue staining of ducts (dotted arrows) and corpuscles (arrow heads) on fascia surrounding tumor tissue; right panel, magnified view of left panel; trypan blue did not stain blood vessels. (C) Multiple ducts (dotted arrows) on fascia surface of tumor tissue; right panel, magnified view of left panel; branching of duct and duct enters nearby fat layer (double arrow). (D) Trypan blue technique revealed Bonghan ducts (dotted arrows) along bundle of blood vessels and nerves; right panel, duct along blood vessel; bundle of blood vessels and nerves connect tumor tissue (an arrow) at lower left corner to outside skin. Samples A, B, C, and D from different mice.

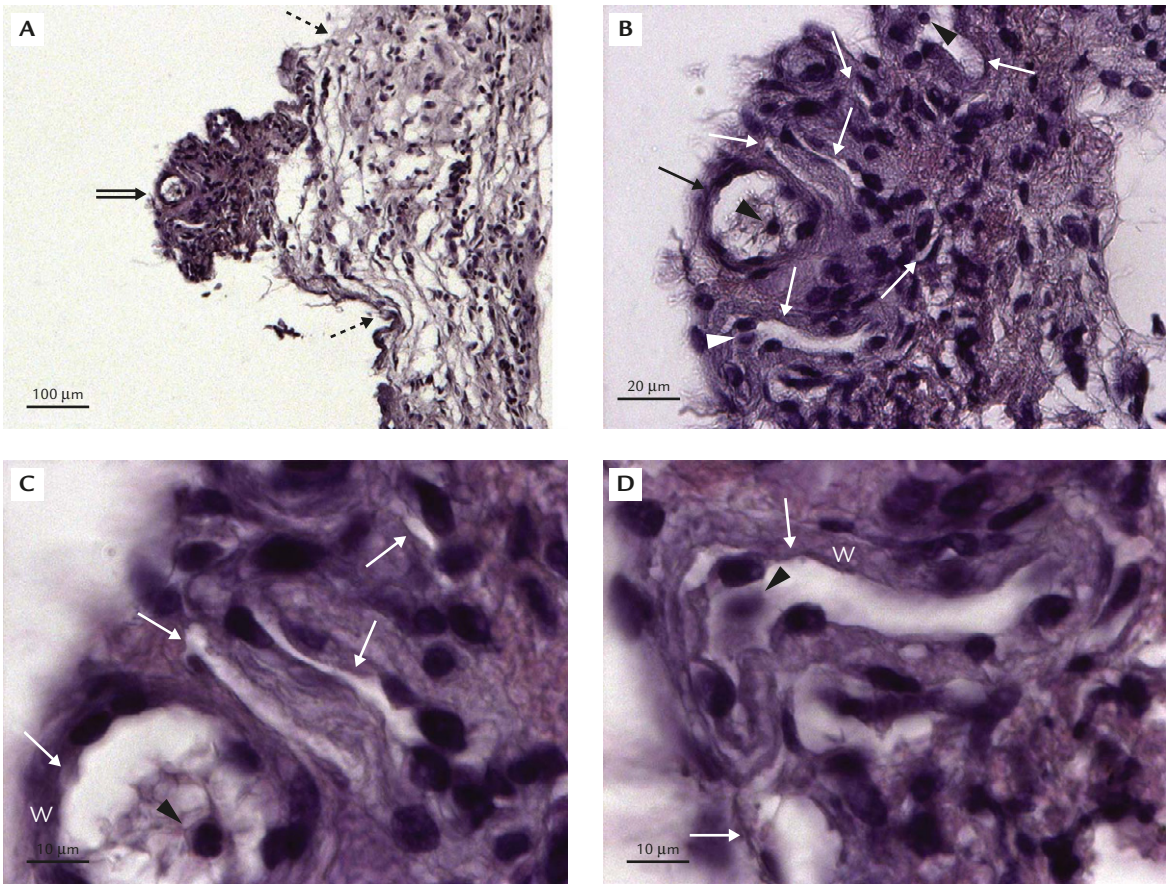

Figure 2 Photomicrographs of cross-sections of Bonghan duct found on the surface of solid mouse tumor with H&E staining. Sample of Figure 1B. (A) Duct (double arrow) located on fascia (dashed arrows) covering solid tumor. (B) Photomicrograph showing several ductules (arrow) in duct, in both cross- and oblique-sections; flowing cells (arrowhead) with round or oval nuclei observed in some duct lumina; nuclei in lumen endothelial cells. (C, D) Duct histological characteristics; high magnification; W, wall of the ductule; arrow and arrowhead, ductule and flowing cells in lumen, respectively. Magnifications: A, 100×; B, 400×; C and D, 1,000×.

tissue to the skin. Thus this could be an effective method for detecting a BHD externally, i.e., by following a blood vessel or a nerve.

A trypan blue positive BHD (Figure 1B) was reexamined histologically with H&E staining (Figure 2), revealing several ductules that appeared in both cross- and oblique-sections. The lumina of the ductules (arrows in Figure 2) contained endothelial cells in the wall of ductules (W in Figure 2) and flowing cells in the space of lumina (arrowheads in Figure 2). The histological nature of the trypan blue positive BHD was distinctively different from the fascia, blood vessels, or lymph vessels, but was quite consistent with the BHDs on internal organ surfaces [15].

A cross-section image of a trypan blue stained BHC identified in a section (Figure 1B) showed the presence of many ductules (Figure 3) and its histological nature was again consistent with other observations involving BHCs on the surfaces of various organs [15].

4. Discussion

A critical goal in verifying the novel metastatic path hypothesis proposed in this article would be to directly show the movement of cancer cells from tumor tissue to other sites via BHDs. Another goal would be to examine whether this route is more efficient than the blood or lymph routes. These goals would require techniques for identifying cancer cells in a BHD and, thus require appropriate markers. The potential use of quantum dot materials engulfed by cancer cells is currently under consideration.

Besides being a potential metastatic path for the spread of cancer cells, BHDs may play other roles in connection with tumor tissues. The liquid flowing in BHDs may play important roles in tumor growth as it contains hyaluronic acid, adrenalin, noradrenalin, albumin, and microcells (or sanals). In addition, a proteomic analysis of tissues and liquid from BHDs on rabbit intestinal surfaces [22] revealed a chemical

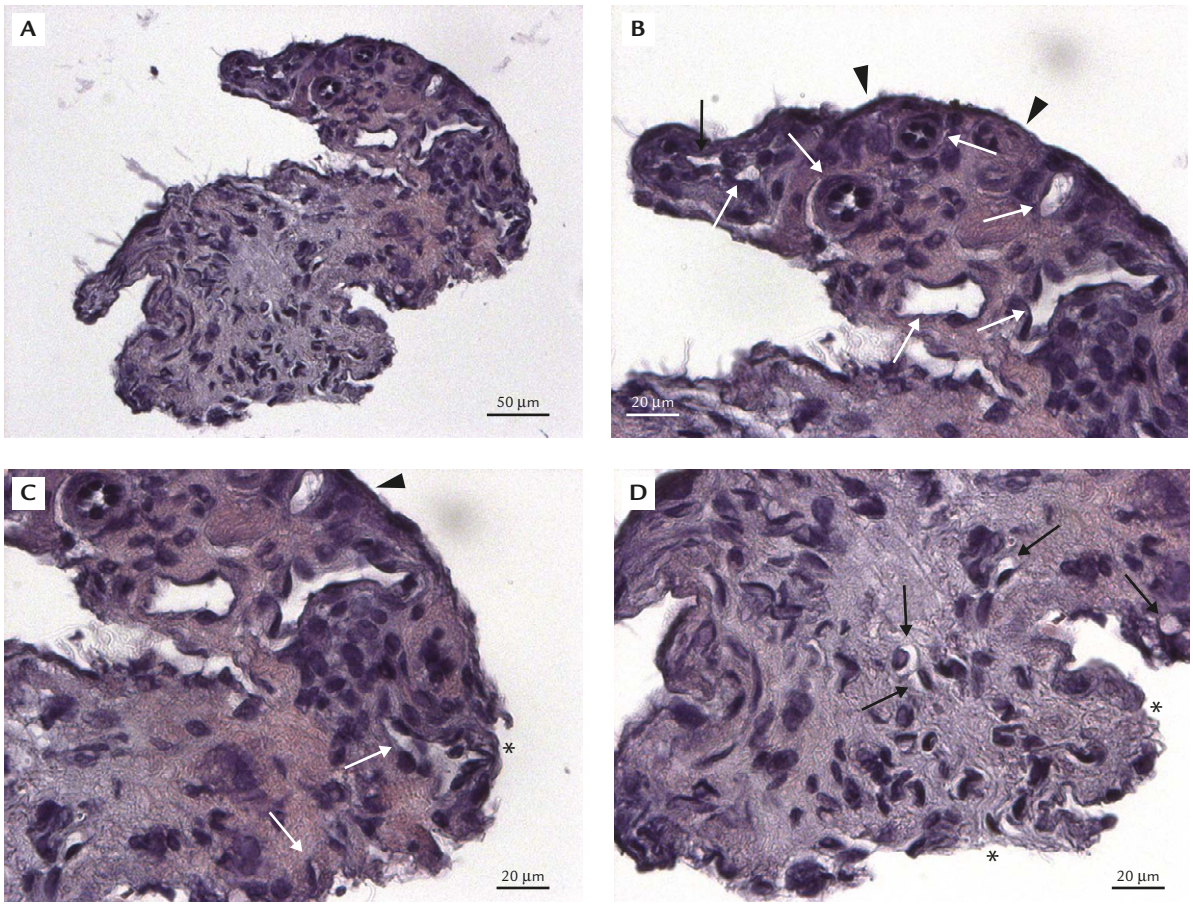

Figure 3 Photomicrographs of Bonghan corpuscle cross-sections. Sample from Figure 1B. (A) Light microscope image of general corpuscle histology. (B–D) Magnified images of Figure 3A; presence of many ductules (arrow) in corpuscles, appear in cross- and oblique-sections; ductule diameter from 5–50 μm; parts of corpuscle boundary surrounded by external connective tissue membrane (arrow heads); parts not (asterisks). Magnifications: A, 200× and B–D, 400×.

composition similar to that usually associated with stem cells [23,24], cancer cells [25], and differentiated myeloid cells [26]. These cells, with vigorous proliferative abilities, show a similar abundance of carbohydrate- or energy-related processes [23–26]. Tumor tissues are likely to take advantage of the nutrients supplied through BHDs.

Another hypothetical significance of the Bonghan circulatory system in relation to tumor tissues is its potential use as a drug delivery path for anticancer medicine or even as a route for acupuncture treatment because the BHD is an anatomical structure of acupuncture collaterals. A host of items, such as migration, invasion, relation to angiogenesis, proliferation, and growth of cancer tissue, have immediate importance in connection with the Bonghan system.

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