Tumor hyperthermia research progress and application prospect in tumoroids (Review)

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Abstract. Tumor hyperthermia is the fifth tumor treatment method after surgery, chemotherapy, radiotherapy and biological therapy, and is also one of the important adjuvant treatment methods for tumors. Hyperthermia can not only directly eliminate tumor cells, but also stimulate the antitumor immune response of the body, and improve the sensitivity of tumor tissues to radiotherapy and chemotherapy. An organoid is a tissue-specific cell cluster formed by 3D culture of various types of cells derived from target organ stem cells, which can reproduce the functions of target organs *in vivo*. At present, the research models of hepatocellular carcinoma (HCC) *in vitro* are mainly 2D culture cell line models, and there is no clinical report on tumor hyperthermia using HCC tumoroids. It was hypothesized that this will be a promising research direction.

Contents

1. Introduction

Tumor hyperthermia is a type of physical therapy that heats up the local tumor focus in the body of the patient to a certain extent through various technologies and methods of non-ionizing radiation to eliminate tumor cells (1). Hyperthermia is the fifth tumor treatment method after surgery, chemotherapy, radiotherapy and biological therapy, and it is also one of the important adjuvant treatment methods for tumors (2). Hyperthermia can not only directly eliminate tumor cells, but also stimulate the antitumor immune response of the body to improve the sensitivity of tumor tissues to radiotherapy and chemotherapy (3). According to the different body parts targeted, hyperthermia can be divided into local hyperthermia, whole body hyperthermia and body cavity perfusion hyperthermia. It is different from the therapeutic mechanism of ultrahigh temperature hyperthermia-induced protein denaturation, such as radiofrequency ablation and microwave ablation. Hyperthermia can directly induce necroptosis or apoptosis of tumor cells by using a warm temperature of 39-45˚C. It can also inhibit tumor growth by destroying tumor blood vessels. At the same time, hyperthermia can activate the response of immune cells and cytokines of the immune system of the body to regulate the immune state of the tumor microenvironment (4). Hyperthermia increases the sensitivity of cancer cells to therapeutic agents, induces direct cytotoxicity, triggers anticancer immune responses and improves drug delivery of various chemotherapeutic agents (4). In the early stages of tumors, the efficacy of chemotherapeutic drugs can be effectively improved by the concomitant administration of heat therapy and chemotherapy. The most appropriate treatment should be chosen, considering the genetic characteristics of the tumor. However, personalized medicine also requires an understanding of the biophysical characteristics of the tumor. Imaging of these features permits rational integration of thermal therapy into treatment regimens to modulate these biophysical features during radiotherapy sessions to enhance the effects of radiotherapy and chemotherapy, and to provide the best possible treatment regimen for the individual patient. During tumorigenesis, a series of changes in glucose, fatty acid, amino acid and cholesterol metabolism occur in tumor cells, which participate in the process of cellular carcinogenesis and constitute part of the underlying mechanism of tumor formation. Thermotherapy can widely affect the metabolism of numerous substances in the treatment of tumors, avoiding the disadvantage of drugs, which can only regulate the metabolism of one substance or one class of substances, meaning the therapeutic effect is not maximized.

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2. Mechanism of hyperthermia

Direct elimination effect. Hyperthermia can not only cause direct irreversible damage to tumor cells, but also effectively inhibit tumor angiogenesis (1). The vascular growth of tumor tissue is deformed and the structure is disordered, the capillaries of tumor tissues are not well developed, twisted and compressed, and a large number of blood sinuses are formed. After heating, it is equivalent to forming a heat storage, causing the temperature of the tumor to be 3-5˚C higher than that in other normal tissues (3). At this time, the vascular resistance and blood viscosity in the tumor are markedly increased, leading to the formation of capillary network thrombosis, subsequently inhibiting the formation of tumor neovascularization, causing tumor cells to become ischemic and necrotic. Hyperthermia can also inhibit the synthesis of proteins, DNA and RNA in tumor cells, inhibiting tumor cell proliferation and leading to cell death. Thermal therapy improves blood flow and oxygenation to the tumor to counteract the deprivation of oxygen and nutrients as well as the decrease in pH caused by poor angiogenesis in malignant tumors. Heat therapy blocks DNA repair enzymes to further support the cytotoxic effects of radiation-induced DNA breaks. Exposure to high temperatures leads to alterations in chromatin structure, which reduces the accessibility of DNA repair mechanisms and leads to perturbations in the repair of DNA double-strand breaks, and heating can indirectly lead to DNA base modifications, including oxidized base damage, basic DNA sites, cytosine deamidation and reactive nitrogen species, induced by heat treatment. Thermotherapy causes protein aggregation, which blocks cell cycle progression in the G_2/M phase. It induces apoptosis via extrinsic and intrinsic pathways (4). Thermotherapy can change the shape of cells, including rounding or flattening, depending on the cell type, and the change in cell shape is accompanied by a reorganization of cytoskeletal elements (3). Generally, microfilaments are rearranged in round cells, while microtubules and/or waveform protein networks are changed in flat cells. Treatments that prevent cytoskeletal reorganization may also enhance cytotoxicity. Thus, cytoskeletal reorganization is not the cause of heat shock or stress, but is part of the heat shock response aimed at cell survival. The high temperatures of thermotherapy lead to disruption of integrin-mediated assembly of the actin cytoskeleton and may disrupt other integrin-mediated signaling pathways.

Necroptosis. Yonezawa *et al* (5) revealed that hyperthermia at 43˚C could induce apoptosis of tumor cells, while necrosis was induced at 44˚C. One of the important characteristics of cell necrosis different from apoptosis is the destruction of the cell membrane. The destruction of the cell membrane can release a large number of cell contents into the tumor microenvironment, causing a systemic inflammatory response and immune response, activating T cells and inducing a specific antitumor immune response (4,6).

Apoptosis. Apoptosis involves a series of irreversible changes caused by caspase family members activated by some pro-apoptotic stimuli, such as endoplasmic reticulum stress and reactive oxygen species (ROS). Morphological features of cell apoptosis include DNA degradation in cells, pyknosis and fragmentation of nuclei, and formation of apoptotic bodies; however, the cell membrane remains intact and does not manifest as a secondary inflammatory reaction (7,8). Heat stress that induces apoptosis also leads to the accumulation of autophagic vesicles and an increase in autophagic flux, which has a survival-promoting effect. *In vivo* and *in vitro*, inhibition of autophagy markedly sensitizes tumor cells to heat-induced apoptosis. The endoplasmic reticulum stress pathway serves a role in heat stress-induced activation of cellular autophagy.

Ferroptosis. Ferroptosis was first proposed by Dixon *et al* (9) in 2012. It is an iron-dependent and peroxide-driven form of cell death. Different from apoptosis, necrosis, autophagy and other cell death modes, ferroptosis is caused by lipid peroxidation and accumulation of ROS. Iron ions in cells react with unsaturated fatty acids highly expressed on cell membranes to produce excessive ROS via the Fenton reaction. Once the ability of the cell to resist oxygen is exceeded, ROS will undergo an oxidative stress reaction, damaging the structure of the mitochondria, endoplasmic reticulum, nucleic acids, in the cell, eventually leading to cell ferroptosis (10). It has been reported that the induction of ferroptosis can effectively inhibit the proliferation and metastasis of tumor cells, and can have a synergistic effect with other antitumor drugs, improving the sensitivity to drug therapy (11). Thermotherapy is able to locally trigger a highly immunogenic ferroptosis in one tumor to initiate potent $CD8^+$ T-cell-mediated systemic antitumor immunity that inhibits the growth of another untreated tumor, thereby facilitating the end result of a durable systemic antitumor effect. Ferroptosis, characterized by excessive lipid peroxidation, has been demonstrated to eliminate cancer cells by bypassing resistance to apoptosis/necrosis. Thermal therapy develops energy through intramolecular movement of excited states to enhance inhibition of tumor cells via the ferroptosis pathway.

Activation of the immune system of the body. Hyperthermia can affect the number and activity of T cells, B cells, natural killer cells, dendritic cells, tumor-associated macrophages and other immune cells, and regulates the expression of immune-related cytokines, including TGF-β, IFN-γ, TNF-α and IL-2, to promote the immune response of the body to exert an antitumor immune effect (12).

Inhibition of epithelial-mesenchymal transition (EMT). EMT refers to the transformation of epithelial cells into mesenchymal cells, which is one of the important features of tumor invasion and migration (13). It is mainly manifested as the abnormal reduction of cell adhesion molecule expression, the marked upregulation of vimentin, fibronectin and other cytoskeletal proteins, and the abnormal upregulation of the Snail family of transcription factors related to EMT (14,15). Hyperthermia can effectively reduce the expression of the EMT-promoting gene Mortalin, reducing the proliferation activity and EMT activity of tumor cells to inhibit tumor progression (16,17).

Functions of deubiquitinating enzyme. The ubiquitin proteasome pathway is an important regulatory system of intracellular protein degradation. Previous studies (18-21) have found that the deubiquitinating enzyme serves an important

role in liver cancer. The deubiquitinating enzyme can reverse the ubiquitination degradation process of proteins, and thus, affect the occurrence and development of tumors, including apoptosis and autophagy, tumor signaling pathways, cell cycle regulation and DNA damage. At present, there are a number of studies (18,21) on ubiquitin specific protease (USP) 22, USP7 and CYLD lysine 63 deubiquitinase in the USPs family, and reducing the activity of these enzymes can effectively inhibit the progress of tumors (18). In addition, there is a Warburg effect existed in malignant tumors, that is the glycolytic metabolism abnormally active even the oxygen content of malignant tumor cells is normal, which is characterized by increased glucose uptake and increased lactic acid content; it is an important metabolic feature of malignant tumors. The rapid proliferation of tumor cells requires high glucose and oxygen consumption, resulting in a relative lack of nutrition and promoting the formation of a local hypoxic microenvironment. Physical and chemical conditions, such as hypoxia, low pH and nutrient deficiency, are conducive to the proliferation and metastasis of tumor cells (19). USP can activate the Warburg effect (20), and knockout of deubiquitinating enzyme expression can promote the sensitivity of liver cancer cells to sorafenib and improve the curative effect (21). Thermotherapy can induce the heat shock response in tumor cells, induce the production of heat shock proteins through high temperature, deubiquitinate them, promote apoptosis and induce mitochondrial dysfunction, inhibit tumor cell proliferation and invasion, and exert anticancer effects.

3. Common methods of hyperthermia

At present, most thermal therapy systems operate by exposing a targeted volume of tissue to ultrasound or electromagnetic radiation energy.

Thermal radiation. Thermal radiation is a method that transfers energy to the treatment area through infrared rays, microwaves, ultrasound or other means, and makes the local temperature rise to produce a therapeutic effect (22,23). However, the temperature is unstable during the treatment process, and the scope of treatment is difficult to define accurately, and thus, it is easy to cause iatrogenic harm to patients. When the skin temperature reaches 41-44˚C, the patient will feel burning pain. If the temperature continues to rise, the skin tissue will be damaged to varying degrees. A skin temperature of 50˚C causes second-degree burn wounds, and a skin temperature of 60˚C for >1 min causes third-degree burn wounds. Therefore, it is seldom used clinically (24).

Hypotonic perfusion of the body cavity. Distilled water at 43˚C is often used for body cavity hypotonic perfusion during malignant tumor resection to eliminate residual free tumor cells. Hyperthermia can change the fluidity of the tumor cell membrane, leading to the destruction of the cytoplasm, while the thermal effect can inhibit the synthesis and repair of DNA, RNA and proteins in tumor cells. When tumor cells are in a hypotonic environment, due to the osmotic pressure difference between the inside and outside of the cells, hypotonic fluid can pass through the cell membrane and enter the cells to directly cause tumor cells to swell, break and dissolve. Distilled water at 43˚C can effectively eliminate tumor cells after acting for 10-30 min (22,23). Hyperthermic intraperitoneal chemotherapy, developed on this basis, is a treatment technology that heats the perfusion solution containing chemotherapy drugs to the treatment temperature and infuses it into the body cavity of patients with tumors for a certain period of time to prevent and treat peritoneal cancer and malignant ascites, which is of great significance in the comprehensive treatment of advanced abdominal cancer (24).

Nanoparticle and magnetic fluid materials. In early clinical hyperthermia, it is difficult to accurately control the temperature and locate the heating range. These two problems led to a bottleneck in the development of hyperthermia. At the end of the last century, the emergence of nanoparticle and magnetic fluid materials overcame this technical problem. With the action of an alternating magnetic field, magnetic nanoparticles can convert magnetic field energy into heat energy. The heating effect is obvious and the temperature is easy to accurately control. The magnetic fluid made of magnetic nanoparticles can be ingested by tumor cells and evenly distributed in tumors. By adding an alternating magnetic field, uniform heating of tumors can be achieved in order to eliminate tumor cells more thoroughly and effectively, which overcomes the low thermal efficiency, difficult thermal control and uneven heat distribution in other hyperthermia schemes (25-27). The biological function of magnetic nanoparticles depends on their ability to reach target molecules and cells *in vivo* (28).

Graphene oxide. Graphene oxide is a novel 2D nanomaterial, which has favorable mechanical and photothermal properties, as well as favorable biocompatibility. It has been reported that graphene and its derivatives can be used for photothermal therapy of tumors (29). Under the irradiation of low-power near-infrared lasers, graphene can convert light energy into heat energy to achieve the purpose of hyperthermia. Graphene materials have the characteristics of small size, high light-to-heat conversion efficiency, targeting to tumor sites and carrying chemotherapy drugs. It is a favorable medical material with application prospects (30).

4. Evaluation of the therapeutic effect of hyperthermia

The evaluation of tumor hyperthermia efficacy is based on the Response Evaluation Criteria in Solid Tumors (31). According to the CT or MRI results, for the measurable target lesions, the ratio of the difference between the maximum tumor diameter before and after hyperthermia and the maximum tumor diameter before treatment (for multiple lesions, the sum of each tumor diameter should be calculated) should be determined. Non-target lesions (other than target lesions) should be recorded at the baseline without measurement, and their presence or disappearance should be noted during follow-up. At the same time, the improvement degree of clinical symptoms of patients, test indicators and imaging examination results should be considered comprehensively (2).

Evaluation of four types of tumors. i) Complete response (CR), disappearance of all target lesions; ii) partial response (PR), reduction of total diameter of target lesion by ≥30% compared

with the baseline; iii) stable disease (SD), between PR and progressive disease (PD); and iv) PD, with the minimum value of the sum of target lesion diameters as the reference, the sum of diameters increases by ≥20%; in addition, the sum of diameters must be increased by at least 5 mm or new lesions must have appeared (2). Thermotherapy is an emerging treatment in oncology. It has become an effective modality for cancer treatment, either alone or in combination with other treatments. Its clinical efficacy is related to the temperature reached during treatment, as well as the duration of treatment, and cellular and tissue characteristics. The lack of precise engineering tools can be observed as a major problem for thermotherapy to become a major part of the treatment and should be improved to ensure that elevated temperature levels are correctly maintained, delivered and localized within the tumor area. Insights into localized thermotherapy lead to disruption of cellular structures and cell death without macroscopic temperature increases, and the effectiveness of antitumor therapy can be further enhanced by synergistically combining thermotherapy with other therapeutic strategies, such as chemotherapy, radiation therapy, immunotherapy and photothermal/photodynamic therapy.

Evaluation of body cavity effusion. i) CR, the body cavity effusion disappeared completely and lasted for >4 weeks; ii) PR, ≥50% body cavity effusion reduced <100% and lasted for >4 weeks; iii) SD, body cavity effusion reduced <50% or increased ≤25% and lasted for >4 weeks; and iv) PD, body cavity effusion increased ≥25% (2).

Evaluation of quality of life of patients with malignant tumors. The body weight, pain and general physical condition are evaluated (2).

5. Common research objects of tumor hyperthermia

Tumor cell lines and strains. The cell population propagated by primary cell culture after successful subculture is referred to as a cell line. A culture with special properties or markers obtained from primary cultures or cell lines by selection or cloning is referred to as a cell strain. Tumor cell lines and strains can be used for gene analysis, drug detection, cytokine detection, artificial organ simulation, monoclonal antibody production, tumor occurrence and treatment. There are a number of relevant studies on hyperthermia using tumor cell lines (32,33). However, cell lines and strains cultured *in vitro* lack a stable internal environment, which may lead to some experimental results being inconsistent with the internal conditions, affecting their reliability and limiting their application.

Tumor animal models. Tumor animal models have been widely used in the study of molecular mechanisms of tumor genesis and development (34,35).

Spontaneous tumor animal models. Spontaneous tumor animal models involve tumors occurring naturally in experimental animals without any conscious artificial treatment. The laboratory animals are mostly inbred mice. Due to the different species and strains of laboratory animals, the types of tumor occurrence and incidence rates differ. The biggest advantage of the animal model of spontaneous liver cancer is that it is a disease that occurs completely under natural conditions excluding human factors. Therefore, the occurrence and development of disease are similar to those of human liver cancer. However, the incidence rate is low and unstable, the occurrence time is long, difficult to predict and uneven, and the individual tumor-bearing animals exhibit large differences in terms of animal characteristics, including sex, weight and tumor occurrence time, and tumor characteristics, including size, number and location. Therefore, its application scope is limited (36).

Transgenic tumor animal models. These are animal models that use genetic engineering technology to introduce or knock out specific genes in animals, affect the expression of animal traits and produce stable genetic modifications. These are also animal models with genetic characteristics and limited to specific genetic characteristics or artificial genetic modification without being widely applicable.

Animal models of carcinogen-induced tumors. Genotoxic carcinogens, including diethylnitrosamine, carbon tetrachloride, aflatoxin and thioacetamide, can directly react with DNA to destroy DNA sites and induce DNA damage to cause cancer. Non-genotoxic carcinogens, including phenobarbital and fibric acid, do not react directly with DNA, but induce tumorigenesis by controlling cell proliferation, apoptosis and differentiation. Although the induced tumor animal models are similar to the natural tumor development process, they have the disadvantages of a long experimental cycle, large individual differences, high mortality and requiring a large number of experimental animals (37).

Animal models of transplanted tumors. Transplanted tumor animal models have the advantages of a short experimental cycle, small individual differences, low lethality and requiring a small number of experimental animals, and have become the most commonly used tumor animal models in the laboratory (35). According to the different sources of grafts, they can be divided into two types: Cell-derived xenografts (CDXs) and patient-derived xenografts (PDXs) (37). The PDX model is an animal model widely used for drug screening at present. Compared with the CDX model, the PDX model can simulate the tumor microenvironment of primary tumors in an improved way, and maintain the heterogeneity of primary tumors, and has improved clinical significance for the treatment of malignant liver tumors (38).

Organoids and tumoroids. Organoids are clusters of tissue-specific cells with intercellular cell/intercellular matrix interactions, which are formed by 3D cultures of target organ stem cells or various types of cells derived from them. Organoids not only highly retain the homology, gene expression, tissue structure and tissue microenvironment of the target organ *in vivo*, but also conform to the cell formation, cell growth and maturation, cell function, self-renewal, and self-organization of the target organ cells, which can mimic the *in vivo* function of the target organ (39). Organoids have been widely used to simulate the occurrence and development of various diseases and for drug sensitivity detection. Hepatocellular carcinoma (HCC)-like tumors still have a lot of room for development, and combining them with some of the existing methods

may render them more viable for targeted drug sensitivity testing. Examples include genetic testing technologies and the CRISPR-CRISPR associated protein 9 system, especially for the use of targeted drugs and immune checkpoint inhibitors. The combination of genetic testing technologies and tumor-like models could further advance precision medicine. By using gene sequencing to screen for therapeutically sensitive drug targets and then using tumor-like organoids to validate target sensitivity, the accuracy of drug delivery can be improved, resulting in improved clinical outcomes. Combined gene editing techniques allow the introduction of specific mutations in HCC organs. It may be useful to adjust the treatment strategy for patients harboring relevant mutant genes. It would also be useful to understand the relationship between different mutant genes and the development of drug resistance.

Patient-derived tumoroids are derived from tumor tissues from patients. They retain the histopathological, genetic and molecular biological characteristics of the original tumor. They cannot only be used as disease models for clinical research, but also to identify and test novel anticancer drugs. The clinical efficacy can be determined based on drug sensitivity testing using tumoroids. The most important advantage of patient-derived tumoroids is that they retain the molecular and individualized functional characteristics of target organs (40), which can provide a reliable basis for individualized precise treatment of patients with tumors to the maximum extent (41). Tumoroids contain various types of immune cells, including functional tumor-infiltrating lymphocytes, natural killer cells and tumor-associated macrophages. Tumoroids have a high clinical association with response to checkpoint inhibitors. Since tumor cells in the traditional 2D culture model cannot simulate the process of communication between cells and between cells and the microenvironment, tumoroids in 3D culture can more accurately reflect the structure of tumor cells in the tumor microenvironment, reflect the real process of tumor occurrence, and serve a guiding role in tumor treatment target and drug screening (42-44).

The diameter of tumoroids is only 0.1-1 mm, requiring observation under a microscope. Tumoroids are high-quality *in vitro* tumor research objects. To judge whether a treatment method is effective, the cell MTT method is generally selected. Succinate dehydrogenase in mitochondria of living mammalian cells can degrade yellow-green MTT into blue-purple formazan crystals and deposit them in cells, while dead cells have no such function. DMSO can dissolve formazan in cells into purple color. The content of formazan can be measured at a wavelength of 490 nm using a microplate reader, and the cell viability can be determined quantitatively. In addition, Cell Counting Kit-8 (CCK-8) assays are also commonly used. The CCK-8 reagent contains water-soluble tetrazolium salts-8, which is reduced to highly water-soluble yellow formazan products by dehydrogenase in cell mitochondria under the action of 1-methoxy-5-methylphenazine dimethyl sulfate, an electron carrier. The amount of formazan generated is in direct proportion to the number of living cells. The light absorption value measured using an ELISA method at a wavelength of 450 nm can indirectly reflect the number of living cells.

Considering the different characteristics and therapeutic responses of different tumor types, thermotherapy has applications in numerous types of cancer. When treating patients with recurrent, newly diagnosed unresectable or high-risk resectable breast cancer, radiotherapy combined with hyperthermia enhances long-term patient outcomes. Thermal therapy increases tumor oxygenation, decreases DNA repair and improves cure rates for patients with prostate cancer. Advances in stem cell culture have given rise to powerful *in vitro* 3D tissue generation techniques, and tumor-like compounds are able to more accurately encapsulate the structure, specific functions, molecular features, genomic changes, expression profiles and tumor microenvironment of the primary tumor. *In vitro* tumor analogs are an important component in the identification of potential therapeutic targets and novel compounds. Thermotherapy is an emerging method for tumor treatment. There is little research on the use of thermotherapy in the field of carcinoid tumors. Using simulations of the carcinoid tumor microenvironment, the specific mechanism of action of thermotherapy in tumor cells can be studied. HCC is associated with poor overall prognosis and high mortality (45). At present, the main research model of HCC *in vitro* is the 2D culture cell line model, which differs from the original tumor in a number of aspects and does not adequately meet the research requirements. Since 2017, the application of patient-derived tumoroids from patients with HCC has partly solved this problem. HCC tumoroids reproduce the process of tumor growth and development *in vitro*, can maintain cell polarity and individual genetic characteristics for a long time, and have unparalleled advantages compared with 2D cell culture and animal models. However, due to the limitations of medical ethics, safety evaluation and favorable clinical practice (46), the clinical application of organoids has not been widely promoted. At present, HCC tumoroids are mainly used in disease models, novel drug development and drug sensitivity testing. In 2017, researchers planted extrahepatic bile duct cell organoids on hollow cylindrical biodegradable stents to obtain biological bile ducts and use them for the repair of extrahepatic bile ducts in rats (47). In 2021, it was reported that bile duct cell organoids could be used to repair human liver grafts (48). However, there is no clinical report on the application of HCC tumoroids in tumor hyperthermia, which is considered to be a promising research direction.

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Competing interests

The authors declare that they have no competing interests.

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