CYPRUS JOURNAL OF MEDICAL SCIENCES

Review

DOI: 10.5152/cjms.2017.192

Cancer in North Cyprus: 2. Biomedical Research Activities

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This is the second part of a two-part review on the cancer status of North Cyprus. Here, we give an overview of the various areas of research on cancer. There are four main areas of ongoing biomedical investigation. First, monitoring of possible carcinogenic chemical factors in the environment (soil and water) is focused on arsenic. Arsenic has been found to exist broadly over the island at levels above the minimum level advised by the Environmental Protection Agency. At present, the source(s) of environmental arsenic is unclear. Second, pathophysiological mechanisms of breast and prostate cancer are being investigated with a focus on ion channels (particularly, the voltage-gated sodium channel) driving the metastatic process. In particular, the triangular relationship involving the channel, its persistent current component, and tumor hypoxia is being elucidated. Third, a further emerging theme is the association of microbiota with cancer. The possible roles of bacterial infections or microbiota in preventing, treating, or causing several types of cancers are among these projects. Fourth, a range of developments in nanotechnology is being considered. It is concluded that the island offers significant opportunities for internationally competitive, multi-faceted research on cancer.

Keywords: Cancer research, North Cyprus, environment, ion channel, microbiology

INTRODUCTION

Our understanding of cancer processes has increased steadily in the last 20 years, the "post-genomic era". Cancer is primarily an epigenetic disease caused by external factors including environmental carcinogens, diet, and lifestyle. The main cause of death in most cancer cases is metastasis, the process in which tumor cells escape from the primary site, enter the circulation (blood and/or lymph) and spread to distant organs where they lodge and re-proliferate to form secondary tumors. This is responsible for approximately 90% of deaths from cancer. Treatment of metastatic disease is invariably systemic (e.g., employing chemotherapy) and suffers frequently from undesirable side effects, including collateral damage to healthy tissues and limited duration of effectiveness, resulting from multi-drug resistance. Hence, identification of novel biomarkers; targets; and early, definitive, and functional diagnosis is necessary to increase the chance of survival.

Previously, we reviewed the cancer status in North Cyprus (NC) (1). It was concluded that whilst cancer incidence in NC is broadly comparable to the rest of Europe, there are some signs of concern. In particular, the incidence of lung, skin, and liver cancers appeared higher and the average age of incidence was lower for breast and skin cancers in NC compared with the rest of Europe. This raised the possibility that "external" factors could be promoting some cancers. It was suggested that continuous monitoring of the environment and research were necessary to understand the possible cause(s) of cancer on the island.

In this part 2 of the overview, we give an account of the cancer-associated biomedical research being conducted in NC, especially at the Biotechnology Research Centre (BRC) of the Cyprus International University. This research is multi-faceted and both "pure" and "applied" in nature, encompassing four main areas.

Environmental Studies

These are centered on arsenic (As), extending the initial joint studies of Cancer Research Foundation/Kanser Araştırma Vakfı (CRF/KAV) and the Frederick Institute of Technology (FIT). Arsenic is a naturally occurring metalloid, classified as a "Group I" human poison and carcinogen by the International Agency Research on Cancer (IARC). Arsenic present in the environment can reach humans via drinking water, agricultural products/food, dermal contact and inhalation of dust. Chronic As poisoning can lead to a variety of adverse health effects including cancers of skin, lung and bladder in addition to cardiovascular diseases, diabetes and gastrointestinal disorders (2). There are two main sources of environmental As: natural (representing the indigenous geological make-up) and anthropogenic (e.g., mining, agriculture and industrial ac-

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Received: 25.11.2016
Accepted: 01.03.2017

tivities). Drinking groundwater containing even naturally occurring high levels of inorganic As can cause large-scale poisoning. Also, plants grown in contaminated areas can transmit As (and other potential carcinogenic metals) via their roots to aerial parts such as stem, leaves and fruits. It has been shown that tuberous vegetables, such as radish, potato and carrot accumulate higher levels of As compared with leafy vegetables such as spinach, amaranthus, cabbage and even less As is accumulated by fruity vegetables such as tomato, eggplant, cauliflower, and bitter gourd. This is followed by pulses such as lentil and pea (3). Biswas et al. (4), on the other hand, reported significantly higher concentration of As in lentil, pea and spinach compared to all tuberous vegetable types. Such contrasting reports would imply that the dynamics of As absorption (and storage) from soil by vegetation is complex and requires further investigation.

We are extending studies on As, following from the initial findings that potentially high As levels are present on the island (5). Preliminary studies have been conducted on soil and water samples collected from Hisarköy, Esentepe, Beyarmudu, Akdoğan and Kalavaç. Within the scope of these pilot studies, As has been detected in some water samples, suggesting strongly the necessity to continue and extend the analyses.

More generally, recent evidence suggests that incidence of As poisoning from groundwater is rising in some parts of the world (6). Because almost 80% of agriculture in NC is carried out in the Guzelyurt region and these agricultural products are consumed all around the island, a new project has been established focusing on this region. The project, funded by EU, is currently under planning at the BRC and will be conducted within the scope of a PhD project. The first aim of this project is to determine As levels in agricultural soil, irrigation water and plants grown in nine selected villages in the Guzelyurt area. The second aim is to evaluate the possible transmission of As to humans. For this, simple body parts (hair, nail clippings and urine samples) will be collected from residents and tested. Thus, the effect, if any, of various environmental parameters (e.g., pH) on possible transmission of As from soil/water to humans will be assessed systematically.

A further project investigates possible As contamination of milk. Milk is a common part of primary nutrition, essential for child growth and body maintenance. It is also thought to protect adults against infectious and non-communicable diseases. Milk has been proposed to be a "complete food", because it contains crucial supplements including proteins, essential fatty acids, lactose, vitamins, and minerals in balanced proportion. However, milk and dairy products can also accumulate contaminants (including As) and chemical hazards (7). The fatty nature of milk is also an ideal medium for dissolving lipophilic compounds such as pesticides and certain hormones (8). Within the scope of this project, monitoring of milk samples from several villages in the Mesaria region, where dairy farming is intensive, is ongoing.

Pathophysiological Mechanisms of Cancer: Role of Ion Channels

This project builds on the discovery of voltage-gated sodium channel (VGSC) as an accelerating mechanism in metastatic diseases (9). VGSCs are membrane-bound proteins composed of one α-subunit (VGSCα) associated with one or more axillary β-subunits (9). The VGSCα is a 200-260 kDa pore-forming protein (functionally independent of any β-subunit) and comprises four homologous domains, each with six transmembrane segments (9). Nine different VGSCα genes (Navl.l-l.9) are present in higher vertebrates. The channels are traditionally known to play a central role in regenerative electrogenesis in "excitable" cells such as neurons, skeletal muscles, cardiac myocytes, and neuroendocrine cells. More recently, VGSCs have also been found to be functionally expressed in "non-excitable" cells including astrocytes, macrophages, and human carcinoma cells. The latter include breast, prostate, colon, cervical, ovarian, non-small cell lung cancer, small cell lung cancer, lymphoma, mesothelioma, neuroblastoma, and melanoma (9, l0). Importantly, where specifically tested, the predominant VGSC isoforms found were "neonatal" splice variants: Navl.5 (breast and colon cancer) and Navl.7 (prostate cancer) (9). More recently, evidence has also been accumulating that VGSC activity promotes metastasis in animal models *in vivo* (e.g. ll). Thus, VGSCs represent a novel anti-metastatic target. In this regard, this channel offers several advantages: early, functional expression; neonatal nature (distinguishing it from other "adult" VGSCs present in the developed body); and the possibility of targeting it using non-toxic drugs.

Normally, a typical VGSC is activated by membrane depolarization and the channel becomes inactivated within a few milliseconds, thus giving rise to a transient Na^+ current (I_{NaT}). Importantly, a limited number of VGSCs may not inactivate or inactivate and re-open during prolonged depolarization. This occurs frequently under pathological conditions, especially reduced tissue oxygen level (hypoxia), and results in the development of a persistent Na^+ current (I_{NaP}). Although the amplitude of I_{NaP} is <l% that of I_{NaT}, its prolonged nature (≥l s) means high amounts of Na^+ can accumulate inside cells (l2). Such excess Na^+ can disrupt cellular homeostasis, especially the pH and Ca^{2+} regulation (l3). Hypoxic conditions develop in several types of growing solid tumors due to poor and/or altered vascular architecture (l4). Development of hypoxia is crucial to dynamic progression of primary tumorigenesis to metastasis in several human cancers.

As regards the VGSC, hypoxia has been shown to increase I_{NaP} in cardiomyocytes and in neurons (l5). In cardiomyocytes, the increased I_{NaP} and the accompanying enhanced Na^+ influx would result in slowing or reversal of Na^+-Ca^{2+} exchange and intracellular Ca^{2+} overload leading to angina (l6). A similar process has been found to occur in cancer cells and number of I_{NaP} blockers (e.g., ranolazine and riluzole) have been suggested to be potential anti-metastatic agents (l7). One of the aims of this project at BRC, supported by TUBITAK, is to study the triangular relationship involving "hypoxia-VGSC/I_{NaP}-metastatic cell behavior" and the associated signaling mechanisms. A central hypothesis is that I_{NaP} blockers, such as ranolazine, can serve as anti-metastatic drugs.

Microbiology and Cancer

There is increasing evidence that cancer and microbiology overlap significantly. On the other hand, imbalance in the composition of the natural microbiome and occurrence of certain bacterial species (e.g., *Helicobacter pylori*) have been found to promote development of cancer. On the other hand, certain microbes and microbial products can offer promise in developing novel therapeutics against cancer (l8). In particular, bacteria can exhibit preferential replication/accumulation mechanisms

inside the hypoxic micro-environment of growing tumors and can be manipulated for cancer treatment (19). The idea of using bacteria as anticancer ("immunotherapy") agents dates back to ca. 1890 when William B. Coley, a surgeon, observed that a patient with neck cancer showed recovery after developing an erysipelas infection (20).

Here, we focus on the potential utilization of bacteria and bacterial products as anticancer agents.

Human body is colonized by a wide variety of microorganisms called "microbiota", sometimes referred to as the "forgotten organ". Microbiota is composed of bacteria in a commensal, symbiotic relationship with the host. This provides various benefits when healthy and balanced, but diseases, including cancer, can arise if imbalanced. Several studies have demonstrated that "germ-free" mice or mice exposed to a heavy dose of antibiotics responded poorly to classic cancer therapies, suggesting that having a healthy gut microbiome is crucial for effective cancer treatment (21). This effect is thought to be related to the immunomodulatory function of the gut microbiota since our resident microbiome can either escalate inflammation or help tone it down. Other studies point to the direct effect of gut microbes in inducing tumors. Accordingly, immunocompromised mice with normal gut flora developed colon tumors, whereas germ-free mice did not (22). Similarly, *H. hepaticus* infection in immunocompromised mice caused colon cancer (23). Composition of microbiota also seems to play a significant role in cancer development. People with precancerous colorectal adenomas displayed excess *Fusobacterium* and *Porphyromonas* in their feces (24). These bacteria (which commonly occur in the mouth) have thus been established as predictive markers for colorectal cancer development (24). However, the "chicken or egg" question still remains: which one comes first, microbiota deregulation or cancer progression? To address this question, mice were treated with antibiotics and then a carcinogen was administered. These mice grew a smaller number of tumors compared with mice with normal microbiota (24). Furthermore, when microbiota was transferred to germ-free or antibiotic-treated mice, the tumor numbers increased upon carcinogen exposure, suggesting that the gut microbiome modulates colon tumorigenesis potentially by promoting inflammation. Conversely, feeding mice *Bifidobacteria* (found commonly in the gut) reduced melanoma progression, suggesting that presence of healthy gut microbes can reduce the burden of cancer of a distinct organ like skin (25). This bacterium is also known to stimulate body's own defenses and used is for immunotherapy. Similarly, feeding *Bacteroides fragilis* to antibiotic-treated or germ-free mice improved the animals' responses to immunotherapy probably by enhancing T-cell infiltration in tumors (24). These studies, although indicating a complex and perplexing relationship between microbiota and cancer, collectively point to the crucial relationship between a healthy immune system and a balanced gut microbiome for the prevention and treatment of cancer.

Bacterial products as oncolytic tools. Bacterial endotoxins (Lipopolysaccharides) and immune-toxins can be used for both destruction of tumor tissue and development of vaccines (26). Bacteria can also be used as vectors for gene therapy and/or administration of anticancer products (27). Spores of anaerobic bacteria can reach and germinate only in oxygen-poor tumor regions and become activated. Many anaerobic bacterial species such as *Bifidobacteria*, pathogenic *Clostridia*, and *Lactobacilli* have thus been tested for their anti-tumor effects (28). Deletion experiments on *Salmonella typhimurium* identified two genes (*msbB* and *purI*) involved in anti-cancer effects, including effects at secondary sites (28). Several other strains of bacteria such as *Listeria monocytogenes, Escherichia coli, Vibrio cholera*, and *S. cholerasuis* have also been examined as possible anticancer agents (26, 28).

Genetically engineered bacteria for gene therapy. Genetically engineered bacteria can serve as tumor-specific vectors (29). Proteins of interest can thus be expressed in a tumor microenvironment enabling powerful therapies to be applied. Such bacterial proteins include cytotoxic peptides, therapeutic proteins, and enzymes as well as diagnostic tools. For example, "tumor amplified protein expression therapy" (TAPET) using modified *S. typhimurium* has been successfully utilized as a diagnostic imaging tool (30). Also, *S. typhimurium* with mutation in the *cya/crp* gene (which would normally encode proteins regulating cyclic AMP levels) has been developed against liver cancer (31). Further, bacteria can be used to express specific host proteins to target tumors. For instance, TNF-α cloned and expressed in *C. acetobutylicum* and *B. adolescentis* showed inhibition of angiogenesis and decreased tumor growth in mouse models (32).

Prodrug therapy with bacteria. Bacterially directed enzyme prodrug therapy is another approach in which toxic side effects of bacteria can be diminished and bacteria can transform a non-toxic prodrug into a cytotoxic agent at the tumor site (33). For example, cytosine deaminase (converting 5-fluorocytosine to 5-fluorouacil) and nitroreductase (transforming CB1954 prodrug to a DNA crosslinking agent) have been utilized in *in vitro* cancer models using *Clostridium sporogenes* as a vector and have been shown to kill tumor cells (33). Similarly, another study showed that intratumoral injection of an attenuated strain of *C. novyi* reduced tumor volume in several preclinical animal models as well as in one phase I study on a human with metastatic disease (27). *Salmonella* has also been used as a vector in this case for cytosine deaminase and nitroreductase which exhibited anti-cancer effects *in vivo* (28).

Bacterial toxins as tumoricidal agents. Bacterial toxins have been tested as possible anti-cancer agents in many models and found to kill cancer cells or alter their activities related to apoptosis, proliferation and differentiation (18, 28). Cytolethal distending toxins (CDTs) and cycle inhibiting factor (CIF) inhibit mitosis, whereas bacterial "cyclomodulins" disrupt host cell metabolism (34). Cell-cycle stimulator (CNF) released by *E. coli* inhibited apoptosis and differentiation of cancer cells (35). In another phase I clinical trial, chimeric toxins, VNP20009 and TAPED-CD, have been successfully tested on cancer patients (36). Using bacterial toxins directed specifically at tumors instead of anti-cancer drugs with broader targets may reduce the side effects of traditional treatments. Furthermore, combinatorial treatments (bacteria or bacterial toxins plus anti-cancer drugs or radiotherapy) could be additionally effective in cancer treatment and be further utilized for immunotherapy.

In summary, various microbiological approaches are effective cancer treatments and can complement existing therapies.

Such approaches include live, attenuated, or dead bacteria, bacterial toxins and genetically engineered bacteria for gene and prodrug delivery as well as immunotherapy use. The symbiotic relationship between the microbiome and the human host has significant implications on various mechanisms ranging from immune regulation to metabolism. In particular, distinct imbalances in oral and gut microbiota have been implicated in various cancers including oral squamous cell carcinoma, esophageal cancer, pancreatic cancer, gall bladder cancer, and colorectal cancer (28, 36). It is important to emphasize, therefore, that a healthy lifestyle that helps maintain well-balanced microbiota can also be a crucial factor in preventing cancer. However, the functional relationship between the microbiome and cancer is complex and substantial effort will be needed to be able to clinically manipulate a patient's resident microbial communities to improve cancer prognosis. More generally, this emphasizes the importance of the microenvironment in tumor progression as well as in the immune component (37).

So far, the research carried out at BRC has focused on the interactions between bacteria (gastrointestinal and environmental species) and tumor cells. We have found that the *E. coli* strain UTI89 isolated from a patient with acute bladder infection was toxic to the strongly metastatic breast cancer MDA-MB-231 cell line (38). Interestingly, this effect depended on type I pili on bacterial cell surface, since piliated bacteria caused a significantly higher level of toxicity compared to non-piliated bacteria, and a non-pathogenic, control strain of *E. coli* (C600), grown under the same conditions (38). No such cytotoxic effect was seen on the weakly metastatic MCF-7 breast cancer cell line (39). Other research is focused on testing the possible anti-metastatic effects of various bacterial species, including *Legionella pneumophila* and its products. This bacterium, an opportunistic pathogen found in water sources and systems, also seems to have cytotoxic and anti-proliferative effects selectively on strongly metastatic cells. Further work using cellular localization/internalization, antibody blocking, and co-localization studies will help determine the mechanisms involved in such bacteria-cancer cell interactions and could lead to novel therapeutic avenues.

Nanotechnology

Nanotechnology is one of the most rapidly developing biomedical research areas worldwide. This technology generates nanometer-sized materials and facilitates molecular modelling and functionality using ultra small materials with novel properties. For example, nano-iron has super paramagnetic properties, whereas macro-iron shows only ordinary magnetic properties (40). Nanotechnology plays an important role in different unique ways and has applications in various areas of medicine, as well as biotechnology and agriculture (41).

The overall aim of nanotechnology in medicine, including cancer, is to find more efficient (faster and cheaper) solutions to clinical problems, including simultaneous diagnosis and therapy and minimization of undesirable side effects (42). Nano-drug delivery systems are also being developed as highly efficient tools for the targeted treatment of cancer (43). Such "nano-carrier" efficiency has improved the therapeutic index of drugs, for example, by improving targeting of tumors in cancer patients. Nanotechnology also reduces drug toxicity and enables the drug to achieve and maintain its therapeutic steady-state level for a longer period by increasing drug solubility and stability. Erdal et al. (44) developed bacterial polyester-based nanospheres for cancer therapy. This study showed that the synthesized nanospheres were selective for cancer cells over healthy cells, thereby enabling targeted therapy (44). In another study, the impact of protein and polymeric nanoparticles (NPs) on breast cancer was investigated (45). These experiments showed that human serum albumin (HSA) NPs were smaller in size compared with (polymer-based) PHB/CMCh nanocarriers. Cellular uptake of drug-loaded NPs was observed, and concanavalin A-coated, etoposide-loaded HAS NPs were more effective on cancer compared with normal epithelial cells and induced selective apoptotic/necrotic effects (45). Silver nanoparticles (AgNPs) have been suggested to be an attractive replacement for antibiotics due to their wider-spectrum antimicrobial activity (46). In this study, we synthesized AgNPs using leaves of *Ficus ingens*. Research also employed UV-visible, fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), and Zetasizer techniques. The antimicrobial activity of the AgNPs was effective at minimum inhibitory concentrations (MICs) of 10 µg/mL for *E. coli* and 20 µg/mL for *S. typhi* and *B. cereus*. Cytotoxicity studies also indicated that the synthesized AgNPs were less toxic to normal cells than to cancerous cells, thereby indicating possible use as novel anti-cancer agents (46).

Nanotechnology is also helping to generate "smart drugs" with reduced side effects compared with conventional therapies. It is expected that nanotechnology will assist in the building of molecular systems with properties of living systems. Such structures could enable regeneration or replacement of body parts lost to infection, accident, or disease (47). A further development is silicon NPs with polymer-like strands holding an anti-cancer drug; these degrade upon entering the cells and discharge the contents (48). Research also aims to develop NPs to target bacteria including gold NPs irradiated with infrared light and iron oxide (FeO) NPs coated with polymer (49).

Another study performed in our laboratory showed that pathogens can be detected using NPs coated with polymeric materials such as chitosan and carboxymethyl chitosan. However, increasing the amount of cross-linker reduced NP production due to formation of denser particles. It would appear, therefore, that cross linking can optimize stabilization of NPs, reducing degradation and increasing surface/volume ratio, thus increasing the capacity of capturing pathogens. Appropriate surface coating with polymeric materials can enable direct immobilization of associated bio-recognition groups on the surface of NPs. The type of functional groups used is an important factor that can affect the stability of magnetic NPs. Thus, this investigation showed that magnetic NPs have a great potential and promising future for pathogen detection. It may ultimately also be possible to modify these NPs for use in cancer management. Indeed, NPs are already serving as MRI and ultrasound image contrast agents, permeation enhancers, and reporters of various types of cell behavior involved in the cancer process, such as apoptosis and angiogenesis. Other ongoing applications of nanotechnology to cancer diagnostics and therapy include the following (50):

- Carbon nanotubes: detection of DNA mutations and disease protein biomarkers;

- Dendrimers: controlled release drug delivery, image contract agents;
- Nanocrystals: improved formulation of poorly soluble drugs;
- Nanoshells: tumor-specific imaging, deep tissue thermal ablation;
- Nanowires: detection of disease protein biomarkers, DNA mutations, and gene expression;
- Quantum dots: optical detection of genes and proteins and tumor visualization.

CONCLUSION

In conclusion, research is already producing results that can throw light onto the cancer status of NC with a view to ultimately improving the relevant conditions. In particular, the levels of As (and other potential carcinogenic heavy metals) in soil, water, vegetation, and the human body need continuous monitoring. More generally, modern "awareness" programs highlighting recent positive developments, such as epigenetics, should encourage and enable people of all ages to learn what to do and what not to do whilst living with cancer in the modern world. In overall conclusion, in the achievement of these aims, the universities of the island would offer significant opportunities to the burgeoning young academic population to carry out internationally competitive multi-faceted research.

Peer-review: Externally peer-reviewed.

Author contributions: Concept - M.B.A.D.; Design - M.B.A.D.; Supervision - M.B.A.D., N.R.; Resource - M.B.A.D., N.R., E.V., E.A., B.A., R.O., O.I., D.K.; Data Collection and/or Processing - N.R., E.V., E.A., B.A., R.O., O.I., D.K.; Analysis and/or Interpretation - M.B.A.D., N.R., E.V.; Literature Search - M.B.A.D., E.A., B.A., R.O., O.I., D.K., E.V, N.R.; Writing - M.B.A.D., E.A., B.A., R.O., O.I., D.K., E.V, N.R.; Critical Reviews - M.B.A.D., N.R.

Acknowledgements: The authors would like to thank the Cancer Research Foundation (CRF)/Kanser Araştırma Vakfı (KAV), the United Nations Office for Project Services (UNOPS), the European Union and TUBITAK for supporting our work on cancer in North Cyprus.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: This research is supported by the Cancer Research Foundation (CRF)/Kanser Araştırma Vakfı (KAV), the United Nations Office for Project Services (UNOPS), the European Union and TUBITAK in the TRNC, and by the Pro Cancer Research Fund (PCRF) in the UK.

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