Chapter 1

Nutrient Synergy: A Novel Approach to Head and Neck Cancer

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Introduction

Head and neck squamous cell carcinoma (HNSCC), which develops in the mucous membranes of the oropharynx, nasopharynx, larynx and hypopharynx, can metastasize to other parts of the body, such as the lymph nodes or lungs leading to poor prognosis. The major cause of death in patients with HNSCC is local invasion and regional and/or distant metastasis. About half of affected individuals do not survive more than five years after diagnosis [1]. HNSCC, the seventh most common cancer worldwide, is diagnosed in approximately 600,000 individuals each year, including about 50,000 in the United States. It usually occurs in males~50- 60 years of age, although the incidence is increasing among younger individuals [1].Head and neck squamous cell carcinomas are correlated with among the most common tumors developed in patients with Fanconi anemia (FA) and the major causes of mortality and morbidity in FA patients [2,3].

Degradation of the extracellular matrix (ECM) by matrix metalloproteinases (MMPs) plays a critical role in the formation of tumors and metastasis and has been found to correlate with the aggressiveness of tumor growth and invasiveness of various cancers [4-6]. In particular, MMP-9 and MMP-2 play key roles in degradation of the ECM and their expression in head and neck carcinoma have been associated with progression and invasion of these tumors [7-10].For example, high MMP-9 expression in sections

of head and neck carcinomas were noted to correlate with aggressive relapses and shortened survival [10].

Selecting Active Compounds for the Nutrient Mixtures

High consumption of fruits and vegetables has been correlated with reduced risk of various cancers, leading to increased attention focused on natural health products for prevention, inhibition and reversal of carcinogenesis [11,12]. In our studies we targeted various mechanisms of cancer by designing specific combinations of different polyphenols and combinations of polyphenols with vitamins and other micronutrients.The efficacy of these nutrient mixtures on tumor growth and cancer invasive parameters was evaluated in various head and neck cancer cell types.

Individual components of the vitamin, polyphenol and micronutrient mixture (NM) were chosen for their potent activity on critical physiological targets in cancer progression and metastasis, such as optimization of collagen structure and stability, inhibition of MMPs, cellular migration and invasion, and induction of apoptosis, as reported in clinical and experimental studies, as noted below. Components of the NM include: Vitamin C (as ascorbic acid,its Mg and Ca salts and ascorbyl palmitate) 700 mg; L-lysine 1000 mg; L-proline 750 mg; L-arginine 500 mg; N-acetyl cysteine 200 mg; standardized green tea extract (80% polyphenol) 1000 mg; selenium 30 µg; copper 2 mg; manganese 1 mg.These compounds play specific functions in the entire mixture. As such, adequate supplies of ascorbic acid and the amino acids lysine and proline are needed for proper synthesis and hydroxylation of collagen fibers and optimal ECM formation and structure [13]. Manganese and copper are also essential for collagen formation. Lysine also acts as a natural inhibitor of plasmin-induced proteolysis to contribute to ECM stability [13,14]. Green tea extract has been shown to control cancer cell growth, metastasis, angiogenesis, and other aspects of cancer progression [15-20]. N-acetyl cysteine and selenium have been reported to inhibit tumor cell invasion and expression of MMP-9, as well as migration of endothelial cells through ECM [21-23]. Ascorbic acid has been reported to exert cytotoxic and antimetastatic actions on malignant cell lines [24-27]; in addition, low levels of ascorbic acid have been reported in cancer patients [28,29]. Arginine is a precursor of nitric oxide (NO); any deficiency of arginine can limit the production of NO, which has been shown to predominantly act as an inducer of apoptosis[30]. By combining these micronutrients, metabolic targets are expanded and simultaneously addressed, maximizing biological impact with lower doses of individual compounds and overcoming absorption barriers.

In addition, we also investigated the effect of a mixture containing multiple polyphenol compounds which target critical physiological targets in cancer progression

Head and Neck Cancer

in order to expand and complement their anti-cancer activity. The mixture of phytonutrients (PB) is composed of: quercetin 400 mg; cruciferex™(proprietary extract from cruciferous vegetables and carrots) 400 mg; turmeric root extract containing 95% curcuminoids 300 mg; resveratrol 50 mg; and standardized green tea extract (80% polyphenols) 300 mg. Curcumin has been reported to affect multiple targets in various cancers, including head and neck squamous cell carcinoma[31].Curcumin has been documented to inhibit cancer cell proliferation, invasion, angiogenesis and metastasis and to induce apoptosis [31]. Cruciferex, a derivative of cabbage, cauliflower, carrot and broccoli, supports anticarcinogenic activity such as detoxification/ excretion of carcinogens, protection against oxidative stress and inhibition of cancer cell proliferation and induction of apoptosis [32].Green tea extract has been shown to modulate cancer progression, including growth, metastasis and angiogenesis [16-18]. Anticarcinogenic effects of resveratrol include modulation of cell proliferation, inflammation, angiogenesis and metastasis and induction of apoptosis[33,34].Cancer preventive effects of quercetin include induction of cell cycle arrest, apoptosis and antioxidant functions [35].

Inhibition of Xenograft Tumor Growth in Head and Neck Cancer by Nutrient Mixtures

We investigated the effect of NM on human HNSCC FaDu [36] and Fanconi anemia HNSCCOHSU-974 [37]

on xenograft tumor growth. The efficacy of the polyphenol combination PB was evaluated on human Fanconi anemia HNSCC OHSU-974 [38]tumor growth. Athymic male nude mice (n=12) were inoculated with 3 x 10^6 FaDu cells subcutaneously and randomly divided into two groups; the Control group was fed a regular diet and the NM group a regular diet supplemented with 0.5% NM. Similarly, athymic male nude mice (n=12) were inoculated with 3x10^6 OHSU-974 cells subcutaneously and randomly divided into a Control group fed a regular diet and the NM group supplemented with 1% NM. In another study, athymic male nude mice (n=12) were inoculated with 3x10^6 OHSU-974 cells subcutaneously and randomly divided into a Control group fed a regular diet and a PB group supplemented with 1% PB. Four weeks later, the mice were sacrificed and their tumors were excised, weighted and processed for histology.

The results of the *in vivo* study of human FaDu xenografts demonstrated significant suppression of FaDu tumor growth (55% reduction in tumor weight) with NM dietary supplementation [36]. In regards to the Fanconi anemia HNSCC OHSU-974 tumor growth, we found that nude mice injected with OHSU-974 cells and subsequently fed a diet supplemented with NM had the growth of OHSU-974 tumors inhibited by 47% (p=0.005) and tumor burden by 50% (p=0.005) [37]. Mice supplemented with 1% PB had tumor growth inhibited by 67.6% (p<0.0001) and tumor burden by 63.6% (p<0.0001) compared to the control group [38]. See Table 1 for summary of results.

Table 1: Effect of NM and PB on tumor xenograft growth in head and neck cancer cells.

Cell Type	Tested Mixture	Effects
FaDu	0.5% NM	55% (p=0.0002) reduction in tumor weight compared to control
FA OHSU-974	1% NM	47% (p=0.005) reduction in tumor growth, 50% (p=0.005) tumor burden
FA OHSU-974	1% PB	67.6% (p<0.0001) reduction in tumor growth and 63.6% (p<0.0001) tumor burden

Inhibition of Cell Growth in Head and Neck Cancer Cell Lines by Nutrient Mixtures

Cell Culture

Human HNSCC FaDu cells were grown in DME media, human FA HNSCC OHSU-974 cells in RPMI, human thyroid SW-579 in Leibowitz medium and human tongue SC-255 in DEM. The cultures were supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 mg/ml) in 24-well tissue culture plates and incubated with 1 ml of media at 37^0 C in a tissue culture incubator equilibrated with 95% air and 5% CO_2. At near confluence, the cells were treated with NM, dissolved in media and tested at 0, 10, 50, 100, 500, and 1000 µg/ml in triplicate at each dose, or with PB dissolved in media and tested at 0, 10, 25, 50, 75 and 100 µg/ml in triplicate at each dose.

Cell Viability

Cell viability was evaluated by MTT assay, a colorimetric assay based on the ability of viable cells to reduce a soluble yellow tetrazolium salt [3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide] (MTT) to a blue formazan crystal by mitochondrial succinate dehydrogenase activity of viable cells. This test is a good index of mitochondrial activity and thus of cell viability. After 24h incubation with tested compounds, cells were washed with phosphate buffered saline and 500 µl of MTT (Sigma #M-2128) 0.5 mg/ml in media was added to each well, plates were covered and returned to the 37^0C incubator for 2h. Following incubation, the supernatant was carefully removed from the wells, the formazan product was dissolved in 1ml DMSO, and absorbance was measured at 570 nm in Bio Spec 1601, Shimadzu spectrometer. The OD_{570} of the DMSO solution in each well was considered to be proportional to the number of cells. The OD_{570} of the control (treatment without supplement) was considered 100%.

NM showed dose-dependent toxicity in FaDu cells, with 53% inhibition of cell growth at 1000 µg/ml NM [36]. OSHU-974 cell toxicity reached 40% with NM at 1,000 µg/ml[37] and 48% in the presence of PB at 100 µg/ml[38]. NM showed dose-dependent inhibition of tongue SC-255 cells with 27% toxicity at 1000 µg/ml [39] but had no appreciable effect on thyroid SW-579 cell proliferation [40].

Effects of Nutrient Mixtures on Apoptosis

The tested cell cultures were washed with PBS and treated with the caspase reagent as specified in the man-

ufacturer's protocol (Molecular Probes Image-IT™ Live Green Poly Caspases Detection Kit 135104, Invitrogen). The cells were photographed under a fluorescence microscope and counted.

Table 2: Effect of NM and PB on cell toxicity and apoptosis in head and neck cancer cells.

Cell Line	Nutrient Mixture	Cell toxicity	Apoptosis induction
FaDu	NM	53% (p=0.0003) at 1000 µg/ml NM	At 100µg/ml: 55% of cells apoptotic (13% in early and 42% in late apoptosis); at 250µg/ml: 76% apoptotic (12% in early and 64% in late apoptosis); at 500 µg/ml: 88% apoptotic (11% early and 77% late apoptosis); at1000µg/ml: most apoptotic (3% in early and 92% in late apoptosis).
FA OHSU-974	NM	40% toxicity at 1,000 µg/ml	N/A
FA OHSU-974	PB	27% (p=0.0003) and 48% (p=0.0004) toxicity at 75 and 100 µg/ml, respectively	N/A
Thyroid SW-579	NM	No significant effect	N/A
Tongue SC-255	NM	17% and 27%(p<0.05) toxicity at 500 and 1000 µg/ml, respectively	At 50 µg/ml: 51% apoptotic (31% early and 21% late stage apoptosis); at 100 µg/ml: 71% apoptotic (33% early and 38% late stage); at 1000 µg/ml: 96% late stage apoptotic

Apoptosis studies were conducted on HNSCC FaDu and tongue SC-255 cells. Dose-dependent induced apoptosis was observed in FaDu cells, with 55% of cells apoptotic (13% in early and 42% in late apoptosis) at 100µg/ml NM, 76% apoptotic (12% in early and 64% in late apoptosis) at 250µg/ml NM,88% apoptotic (11% early and 77% late apoptosis) at 500µg/ml NM and virtually all cells

exposed to 1000µg/ml NM in late apoptosis(3% in early and 92% in late apoptosis) [36].Dose-dependent induction of apoptosis by NM was also found in tongue SC-255 cells with 51% apoptotic cells at 50 µg/ml (31% early and 21% late stage apoptosis), 71% at 100 µg/ml (33% early and 38% late stage) and 96% late stage apoptotic cells at 1000 µg/ml [39].See Table 2 for summary of results.

Modulation of Invasive Parameters in Head and Neck Cancer Cell Lines

Invasion of host tissues is dependent on tumor cell adhesion, cell migration, and proteolytic degradation of the ECM by MMPs [4].As mentioned previously, MMPs, especially MMP-2 and MMP-9 are prognostic for survival and metastatic potential in head and neck cancers. Patel et al. [41] found that latent, active, and total forms of MMP-2 and MMP-9 were significantly elevated in malignant tissue of oral cancer patients compared with adjacent normal tissues and that MMP-2 was correlated with lymph node metastatic development. Kawamata et al. [8] found increased activity of pro-MMP-9 and active MMP-2 in cancer cell nests compared to normal surrounding gingival tissue and significantly higher MMP-2 activity in metastatic cancer cell nests in human oral squamous-cell carcinoma tissues. In examining a group of patients with early stage oral squamous cell carcinoma, Katayama et al. [42] found significantly increased MMP-9 and TIMP-

2 expression in patients who developed regional lymph node and/or distant metastasis than patients without any tumor metastasis that also correlated poorer survival. Reidel et al. [43] found that MMP-9 expression in patients with HNSCC correlated with poor survival of patients, high VEGF expression and higher mean vessel density than MMP-9 negative tumors. Kurahara et al. [44]demonstrated increased expression of MMP –1,-2,-3,-9 and MT1-MMP in invasive and metastatic cases of oral squamous cell carcinoma and a significant loss of ECM.Since proteases, especially MMP-2 and MMP-9, play key roles in tumor cell invasion and metastasis, we investigated the effects of NM in vitro on HNSCC FaDu, FA HNSCC OSHU-974, tongue SC-255 and thyroid SW-579 cells in respect to MMP-2 and MMP-9 secretion and invasion of cells through Matrigel. We also studied cell migration in some of the cells.

Secretion of MMPs

Gelatinase zymography was utilized because of its high sensitivity to gelatinolytic enzymatic activity and ability to detect both pro and active forms of MMP-2 and MMP-9. Gelatinase zymography was performed in 10% Novex pre-cast SDS polyacrylamide gel in the presence of 0.1% gelatin under non-reducing conditions. Cell culture media (20 µl) were mixed with sample buffer and loaded for SDS-PAGE with Tris glycine SDS buffer, as suggested by the manufacturer (Novex). Samples were not boiled

before electrophoresis. Following electrophoresis the gels were washed twice in 2.5% Triton X-100 for 30 min at RT to remove SDS. The gels were then incubated at 37°C overnight in substrate buffer containing 50mM Tris-HCl and 10mM $CaCl_2$ at pH 8.0 and stained with 0.5% Coomassie Blue R250 in 50% methanol and 10% glacial acetic acid for 30 minutes and destained. Protein standards were run concurrently and approximate molecular weights were determined by plotting the relative mobilities of known proteins.

Zymography showed the presence of MMP-2 and PMA-induced MMP-9 secretion in FaDu cells; NM inhibited secretion of both MMPs in a dose-dependent manner, with virtual total inhibition at 1000 µg/ml [36].In FA OSHU-974 cells zymography revealed MMP-2 and PMA-induced MMP-9 secretion, which was also suppressed by NM in a dose-dependent manner, with a virtual inhibition of both at 500µg/ml [37]. In these cells PB achieved total block of secretion of the MMPs at 50 µg/ml [38]. Thyroid SCC-255 cells only secreted MMP-2 which was inhibited by NM with virtual total block at 1000 µg/ml [40]. Tongue SW-579 demonstrated secretion of MMP-2 and PMA-induced MMP-9. Both these MMPs were blocked by NM at 500 µg/ml [39].See Table 3 for summary of results.

Table 3: Effective concentrations of NM and PB on inhibition of invasive parameters in head and neck cancer cell lines.

Cell Line	Nutrient Mixture	100% inhibition of MMP-2	100% inhibition of MMP-9	100% Matrigel cell invasion	100% inhibition of cell migration
FaDu	NM	1000 µg/ml	1000 µg/ml	1000 µg/ml	N/A
FA OHSU-974	NM	500 µg/ml	500 µg/ml	1000 µg/ml	250 µg/ml
FA OHSU-974	PB	50 µg/ml	50 µg/ml	50 µg/ml	50 µg/ml
Thyroid SW-579	NM	1000 µg/ml	N/A	500 µg/ml	N/A
Tongue SC-255	NM	500 µg/ml	500 µg/ml	1000 µg/ml	250 µg/ml

Invasive Properties of Cancer Cells

Invasion studies were conducted using Matrigel™ (Becton Dickinson) inserts in 24-well plates. Cells were suspended in medium, supplemented with specified nutrients, seeded on inserts in wells. Plates with inserts were then incubated in a culture incubator equilibrated with 95% air and 5% CO_2 for 24 h. After incubation, media from wells were withdrawn and cells on the upper surface of inserts were gently scrubbed away with cotton swabs. Cells that had penetrated the Matrigel™ membrane and migrated onto the lower surface of the Matrigel™ were stained with Hematoxylin and Eosin and visually counted under the microscope.

NM significantly inhibited FaDu invasion through Matrigel™ with total block at 1000 µg/ml [36]. Invasion of thyroid cells was inhibited by NM at 50, 100, and 500 µg/ml by 42%, 63%, and 100%, respectively [40]. Tongue cell invasion was inhibited by NM dose-dependently with 100% block at 1000 µg/ml [39].NM inhibited OHSU-974 cell invasion through Matrigel™ in a dose-dependent manner with a complete block at 1000 µg/ml [37], while PB achieved total block at 50 µg/ml [38].See Table 3 for summary of results.

Cell Migration

To study cell migration, a 2-mm wide single uninterrupted scratch was made from the top to bottom of culture plates of tested cells grown to confluence. Culture plates were washed with PBS and incubated with NM in medium and tested at 0, 50, 100, 250, 500 and 1000 µg/ml, in triplicate at each dose for 24h. Cells were washed with PBS, fixed and stained with H&E and photomicrographs were taken.

Studies demonstrated total block of cell migration of FA OHSU-974 by NM at 250 µg/ml [37] and by PB at 50 µg/ml [38]. Tongue SC-255 cell migration was totally blocked by NM at 250 µg/ml [39].See Table 3 for summary of results.

Conclusion

The review of our various studies investigating the efficacy of the vitamin/micronutrient and polyphenol mixture on various head and neck cancers show significant suppression of tumor growth, cell proliferation, MMP-2 and -9 secretion, cell migration and Matrigel invasion of these cancer cells. These results indicate that these non-toxic nutrient mixtures have therapeutic potential in prevention and treatment of head and neck cancers.

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Head and Neck Cancer

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Head and Neck Cancer

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Head and Neck Cancer