ORIGINAL RESEARCH

Inhibitory Effects of a Novel Nutrient Mixture on MMP Secretion and Invasion on Human Thyroid Cancer Cell Line SW 579

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ABSTRACT

Thyroid cancer is the most common endocrine malignancy. Mortality from thyroid cancer results from tumor invasion with local and distant metastases. Degradation of extracellular matrix is the hallmark of metastasis and is mediated by matrix metalloproteinase (MMP) enzymes. A novel Nutrient Mixture (NM) containing ascorbic acid, lysine, proline, and green tea extract has exhibited significant anticancer activity in other cancer cell lines. In this study, we investigated the effects of NM on a thyroid cancer cell line on proliferation (MTT Assay), MMP secretion (Gelatinase-Zymography), Matrigel™ invasion, and morphology (H&E). Zymography demonstrated that NM inhibited MMP-2 secretion, with virtually total inhibition at 1000 mg/mL. Matrigel™ invasion was inhibited at 50, 100, and 500 mg/mL by 42%, 63%, and 100%, respectively. NM was nontoxic to thyroid cancer cells below 500 mg/mL, and H&E staining did not show morphological changes.

CONCLUSION

NM significantly inhibited critical steps in cancer progression by blocking MMP-2 enzymes and Matrigel™ invasion.

KEY WORDS

Thyroid cancer, MMPs, Matrigel™ invasion, nutrients, green tea extract, ascorbic acid, lysine.

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INTRODUCTION

Thyroid cancer is the most common of all endocrine cancers. American Cancer Society estimates approximately 33,550 new cases of thyroid cancer will be diagnosed in the United States in 2007, and approximately 1530 deaths are estimated due to thyroid cancer. Some of the risk factors for developing thyroid cancer are: white race, female gender, low intake of iodine, and previous radiation exposure. Thyroid cancer will constitute 4% of all female cancers in 2007. Women between ages of 40 and 70 are at higher risk; however, women as young as 19[1] have been reported to be diagnosed with this disease. The hormone estrogen is thought to increase the rate of thyroid cancer in women.[2,3,4] Radiation, especially therapeutic radiation for Hodgkin's disease in childhood, increases chances of developing thyroid cancer later in life. Lowering the doses of radiation has not decreased this risk.[5]

Thyroid cancers are divided into three categories: differentiated, medullary, and anaplastic cancers. Well-differentiated thyroid cancers (papillary and follicular) grow slowly, are rarely fatal, but may recur. Medullary cancers developing from C cells of the gland metastasize faster and are resistant to radioactive iodine. Anaplastic thyroid cancers are relatively uncommon, yet the most dangerous, of malignant tumors, and have the worst prognosis.

The accepted methods of treatment for all types of thyroid cancers include surgery, radioactive iodine, thyroid hormone supplementation, external beam radiation therapy, and chemotherapy.[6] The best suggested approach by the American Cancer Society is a combination of two or more of these methods. In most cases, surgery is followed by radioactive iodine treatment, with or without supplemental thyroxin. Chemotherapy has very limited use and is only used as palliative care.[7] Despite this combination approach, anaplastic

thyroid carcinoma responds very poorly and has uniformly dismal prognosis with median survival of 3 to 7 months.[8, 9]

This indicates that at this point there is no radical cure available for thyroid cancer, and multiple treatment combinations are tried in the hope of achieving maximum survival.[10] The standards of treatment, including surgery, radiation, chemotherapy, or even a combination of them, have not addressed the aspect of metastases, marking these approaches less effective at improving survival in the majority of cases.[11,12] Clearly there is a need for a new, safer, and more effective approach for thyroid cancer.

Previous studies have shown that proteolytic degradation of extracellular matrix (ECM) is the key factor in stromal invasion of the tumors and eventual metastasis. Matrix metalloproteinases (MMPs) are a group of calcium-dependent, zinc-containing endopeptidase enzymes that are responsible for the tissue remodeling and degradation of the extracellular matrix, including collagen and elastin. MMPs are excreted by a variety of connective tissue cells.[13,14] These MMPs participate in normal tissue remodeling, such as embryonic development, angiogenesis, and wound healing. Studies have shown that the aggressiveness of the cancer indicated by invasiveness, grade, and stage, is highly correlated with the expression of MMPs.[15] Though several proteolytic enzymes are postulated to play a role in this process, MMPs, especially MMP-2 and MMP-9, are identified as the most important.[16]

Rath and Pauling postulated that nutrients such as lysine and ascorbic acid (Vitamin C) could act as natural inhibitors of ECM proteolysis and, therefore, by stabilizing the connective tissue, have the potential to modulate tumor growth and metastasis.[17] These nutrients can utilize their antitumor potential through several mechanisms, including the inhibition of MMPs, as well as strengthening of connective tissue surrounding cancer cells, which is also known as "Tumor encapsulating effect". Our previous studies have also confirmed this approach.[18, 19]

In our current study, we have investigated the antitumor potential of an in vitro Nutrient Mixture (NM) on thyroid cancer in the SW 579 cell line by measuring cell proliferation, modulation of MMP secretion, cancer cell invasive potential, and morphology. The Nutrient Mixture (NM) is a combination nutrients formulated to target the key physiological pathways in cancer progression and metastasis.

MATERIALS AND METHODS

2.1 Cell Culture

Human Anaplastic Thyroid Carcinoma Cell line SW 579, obtained from ATCC (American Type Culture Collection, Rockville, MD), was grown in Leibowitz medium with 10% feta bovine serum, penicillin (100 U/mL), and streptomycin (100 mg/mL) in 24-well tissue culture plates (Costar, Cambridge, MA). Cells were incubated with 1 mL of medium at 37°C in a tissue culture incubator equilibrated with 95% air and 5% CO_2. At near confluence, the cells were treated with Nutrient Mixture (NM) dissolved in media at 0, 10, 50, 100, 500, and 1000 mg/mL. The plates were then returned to the incubator.

2.2 MTT Assay

Cell proliferation was evaluated by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay, a colorimetric assay based on the ability of viable cells to reduce a soluble yellow tetrazolium salt (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 incubating for 24 hours, the cells were washed with phosphate-buffered saline (PBS) and 500 ml of MTT (Sigma Catalog No. M-2128), 0.5 mg/mL media was added to each well. The plates were covered and returned to the 37°C incubator for 2 hours, the optimal time for formazan product formation. Following incubation, the supernatant was carefully removed from the well, the formazan product was dissolved in 1 mL DMSO (Dimethyl sulfoxide), 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 to be 100%.

2.3 Gelatinase Zymography

MMP secretion in conditioned media was determined by gelatinase zymography. Gelatinase zymography was performed in 10% polyacrylamide precast Novex gel, sodium dodecyl sulphate (Invitrogen Corp.), in the presence of 0.1% gelatin under non-reducing conditions. Culture medium (20 ml) was loaded, and sodium dodecyl sulphate (SDS)-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed with Tris-Glycerine SDS buffer as described by the manufacturer (Novex). Samples were not boiled before electrophoresis. After electrophoresis, the gels were washed with 5%_Triton X-100 for 30 minutes at room temperature to remove SDS. The gels were then incubated at 37°C overnight in the presence of 50 mM Tris-HCl, 5 mm $CaCl_2$, 5 mM $ZnCl_2$ at pH 7.5, stained with Coomasie Blue R 0.5% for 30 minutes, and destained. Protein standards were run concurrently, and approximate molecular weights were determined by plotting the relative mobilities of known proteins.

2.4 Matrigel™ Invasion Studies

Invasion studies were conducted using Matrigel™ (Becton-Dickinson) inserts in 24-well plates. Suspended in medium, human thyroid cancer cells SW 579 were supplemented with nutrient, as specified in the design of the experiment and seeded on the insert in the well. Thus, both the medium on the insert and in the well contained the same

supplements. The plates with the inserts were then incubated in a culture incubator equilibrated with 95% air and 5% CO_2 for 24 hours. After incubation, the media from the wells were withdrawn. The cells on the upper surface of the inserts were gently scrubbed away with cotton swabs. The cells that had penetrated the Matrigel™ membrane and had migrated onto the lower surface of the Matrigel™ were stained with hematoxylin and eosin and visually counted under the microscope.

2.5 Morphology

Morphology of cells cultured for 24 hours in the test concentrations of NM were evaluated by H&E staining and observed and photographed by microscopy.

2.6 Composition of Nutrient Mixture (NM)

Stock solution of the Nutrient Mixture (NM) prepared for testing was composed of the following: Vitamin C (as ascorbic acid, and as Mg, Ca, and palmitate ascorbate) 700 mg; L-lysine 1000 mg; L-proline 750 mg; L-arginine 500 mg; N-acetylcysteine 200 mg; standardized green tea extract 1000 mg (green tea extract was derived from green tea leaves obtained from US Pharma Lab.)

The certificate of analysis indicates the following characteristics: total polyphenol 80%, catechins 60%, epigallocatechin gallate [EGCG] 35%, and caffeine 1.0%, selenium 30 mg, copper 2 mg, and manganese 1 mg.

The Nutrient Mixture (NM) was formulated based on targeting different physiological processes involved in cancer progression and metastasis. For example, the ECM integrity is dependant upon adequate collagen formation and its stability. In this aspect, ascorbic acid and the amino acids lysine and proline are necessary for the formation and optimum structure of collagen fibers. Manganese and copper are also essential cofactors in the collagen formation process. Collagen stability can be controlled by lysine[17] and also by N-acetylcysteine through its inhibitory effect on MMP-9 activity[20] and invasive activities of tumor cells.[21, 22] Selenium has also been shown to interfere with MMP expression and tumor invasion,[23] as has migration of endothelial cells through ECM.[21] Ascorbic acid has been shown to inhibit cell division and growth through production of hydrogen peroxide.[24] Green tea extract has shown to be a promising agent in controlling angiogenesis, metastasis, and other aspects of cancer progression.[25,26] Because arginine is a precursor of nitric oxide (NO), any deficiency of arginine can limit the production of NO, which has been shown to act predominantly as an inducer of apoptosis, as in breast cancer cells.[26]

Based on the evidence available in literature and our own research, we have postulated that metabolic effects of a combination of ascorbic acid, lysine, proline, green tea extract, arginine, N-acetylcysteine, selenium, copper, and manganese would result from their synergy. For example, we found that a combination of ascorbic acid, lysine, and proline used with EGCG enhanced the anti-invasive activity of 20 mg/mL EGCG to that of 50 mg/mL.[27] Thus, by including N-acetylcysteine, arginine, selenium, manganese, and copper with ascorbic acid, proline, lysine, and EGCG, we could obtain significant reduction in cell invasion at a much lower concentration of EGCG or other components. Also, the combined effects of these individual nutrients on decreasing proliferation of neoplastic cells were superior to the effects of their individual components or when they were randomly combined.[28]

2.7 Statistical Analysis

The results were expressed as means ± SD (Standard Deviation) for the groups. Data was analyzed by independent sample "t" test.

RESULTS

3.1 Cell Proliferation Study

The Nutrient Mixture (NM) had no significant toxic effect on human thyroid carcinoma SW 579 cell proliferation as shown in Fig.1.

3.2 Gelatinase Zymography Study

Gelatinase Zymography study shows only one band corresponding to MMP-2. MMP-9 is not secreted by SW 579 cell line. The expression of MMP-2 was decreased with increasing concentration of NM as shown in Fig. 2A. Densitometry analysis as seen in Fig. 2B shows that with the increasing concentrations of NM from 10, 50, 100, 500, and 1000 mg/ml, the expression of MMP-2 decreased from 107, 97, 85, and 20% respectively to almost complete inhibition at 1000 mg/ml, with R^2 value being 0.8007.

3.3 Invasion studies

The Nutrient Mixture (NM) significantly reduced the invasion of thyroid carcinoma cells through Matrigel™ in a dose dependant fashion, as seen in Fig. 4, with 42% inhibition at 50 mg/mL, 63% inhibition at 100 mg/mL, and 100% at 500 mg/mL (P=0.0002) NM, as shown in Fig. 3.

3.4 Morphology study (H&E staining)

NM showed no effect on morphology of thyroid cancer cell line SW 579 up to 100mg/ml. However, significant changes were seen at higher doses, from 500 to 1,000 mg/mL, as seen is in Fig. 5-A through 5-F.

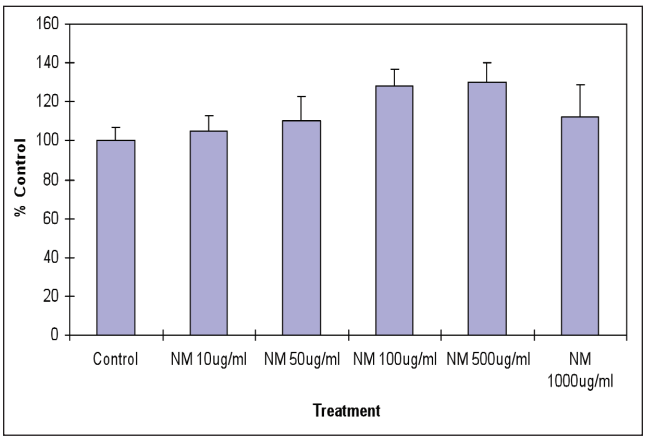
DISCUSSION

In the current study, we analyzed the effects of NM on anaplastic thyroid cancer cell line SW 579. The results indicate that NM is effective in inhibition of Matrigel™ invasion, in a dose dependant manner, by thyroid cancer cells SW 579. In addition, we also noticed that NM decreases MMP-2 secretion by thyroid cancer cells in a dose depen-

dant fashion. NM did not seem to affect the morphology of cells SW 579 below 500 mg/mL. The results obtained with this thyroid cancer cell line corroborate with our previous data evaluating NM effects on other cancer cell lines[29,30,31] proving that the inhibitory actions of the Nutrient Mixture (NM) on MMP secretion and Matrigel™ invasion are similar to most other cancer cell lines.

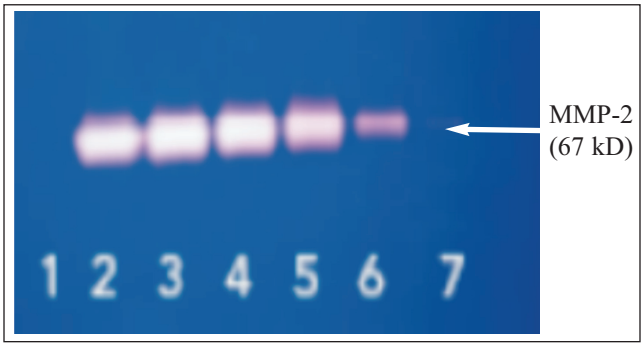
Anaplastic thyroid carcinomas constitute 1 to 5% of all thyroid cancers and are an extremely malignant tumor of the elderly population. Long-standing and well-differentiated thyroid cancers, such as papillary and follicular types, frequently coexist with the diagnosis of anaplastic thyroid cancer in 23 to 90% of the cases, and are also thought to be a predisposing factor for development of anaplastic thyroid carcinoma.[32] Prolonged goiter with Thyroid Stimulating Hormone (TSH) treatment may be responsible for such changes.[33] Anaplastic thyroid carcinoma is assumed to be one of the most destructive of all human malignancies. Fatalities attributed to this cancer are due to local and distant metastasis. Local invasion in the trachea and esophagus is as dangerous as distant metastasis in lungs, bones, and brain. Therefore, controlling this process of metastatic is the topmost priority. Many studies have indicated the importance of MMP enzymes in tissue remodeling and tumor progression through extracellular matrix (ECM) degradation. Of all MMP types, MMP-2 and MMP-9 are indicated to cause the highest level of collagen IV destruction. Collagen is the main component of cellular basement membrane and therefore plays a critical role in tumor progression and invasion. Researchers have shown, especially in papillary thyroid cancer, that plasma Vascular Endothelial Growth Factor (VEGF) and MMP-9 are significantly increased and are very good indicators of its metastatic potential.[16] It has also been proven that MMP-2 and its tissue inhibitors play a vital role in the pathogenesis

Figure 1. Effect of NM on thyroid cancer cells SW 579 cell proliferation: MTT Assay 24 hours.



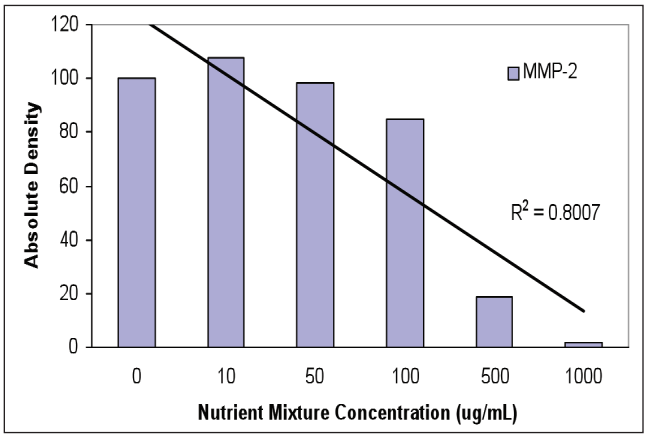
The nutrient mixture (NM) had no significant toxic effect on human thyroid carcinoma SW 579 cell proliferation.

Figure 2. Effect of NM on MMP secretion of thyroid cancer cell line SW 579: 2A- (Legend: 1- Markers, 2- Control, 3-7 NM 10, 50, 100, 500, 1000 µg/ml).



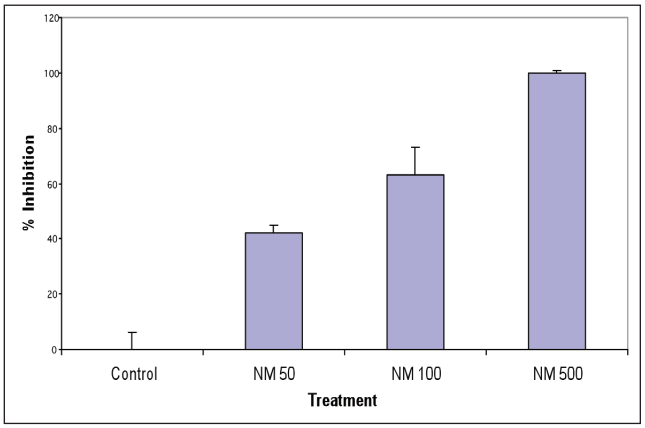
Cells were cultured, challenged with different concentrations of NM and condition media was applied for Zymography.

Figure 2B. Densitometry Analysis: Effect of NM on relative activity of MMP-2 in human thyroid cancer cells SW 579.



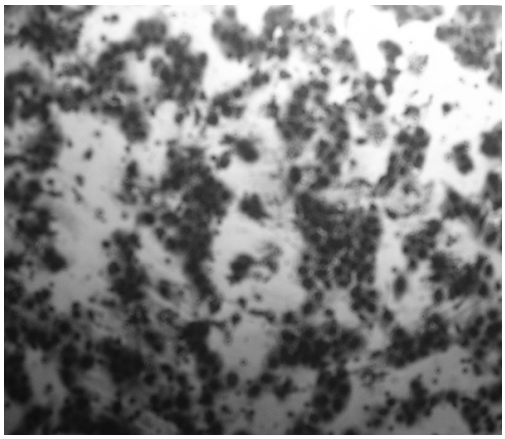
The expression of MMP-2 is decreasing with increasing concentration of NM.

Figure 3. Effect of NM on Matrigel Invasion of thyroid cancer cells SW 579.

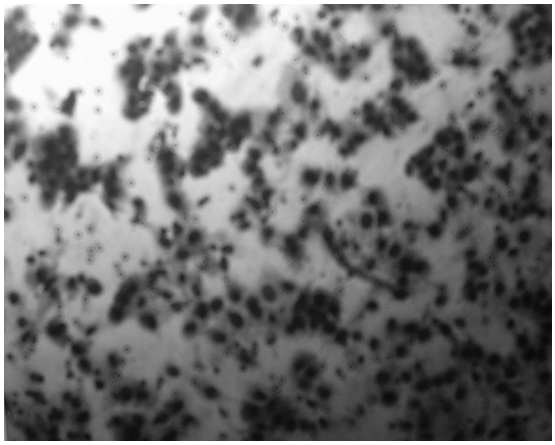


The nutrient mixture (NM) significantly reduced the invasion of thyroid carcinoma cells through Matrigel in a dose dependant fashion.

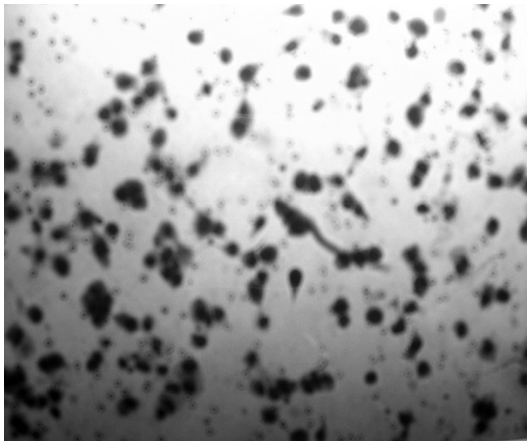
Figure 4. Thyroid Cancer cells SW 579 Invasion Photomicrographs.



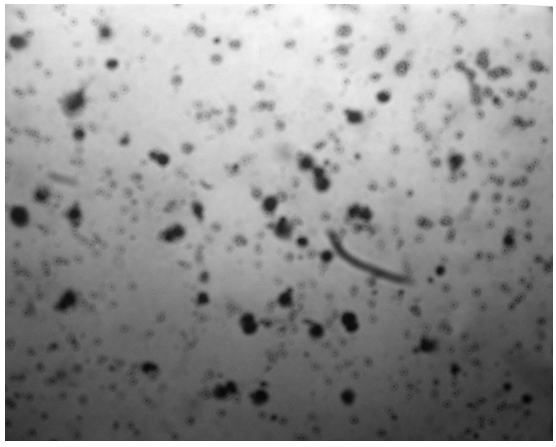
4A - Control



4B - NM 50µg/ml



4C - NM 100µg/ml

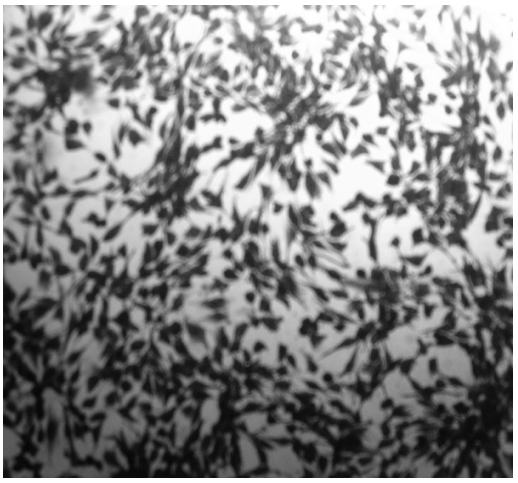


4D - NM 500µg/ml

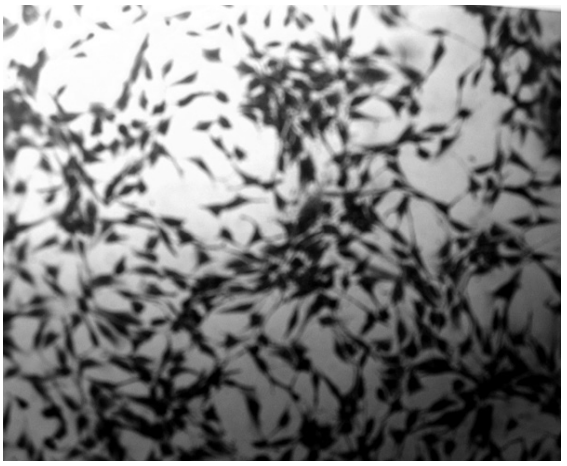
4E - NM 1000µg/ml

The nutrient mixture (NM) significantly reduced the invasion of thyroid carcinoma cells through Matrigel in a dose dependant fashion.

Figure 5. Thyroid Cancer cell SW 579 cell Morphology Photomicrographs (H&E Staining).



5A - Control

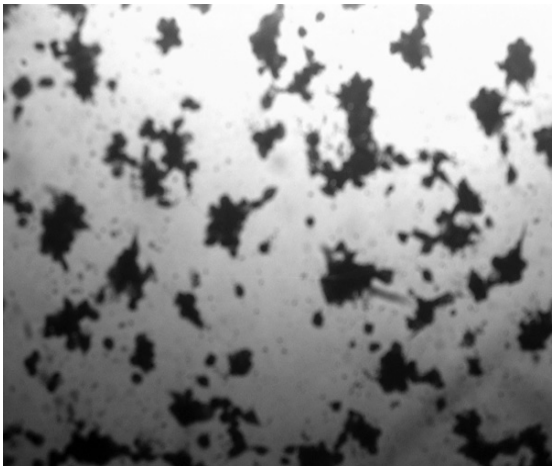


4B - NM 10µg/ml

5C - NM 50µg/ml

5D - NM 100µg/ml

5E - NM 500µg/ml



5F - NM 1000µg/ml

NM was not toxic to thyroid cancer cells below 500 µg/ml

of thyroid cancer.[34] Higher expression of both MMP-2 and MMP-9 is significantly correlated with larger tumor size, lymph node metastasis, higher clinical stage, and increased potential for intra-thyroidal and vascular invasion.[53] Increased MMP-2 secretion can also be used as a diagnostic marker to differentiate papillary versus other thyroid neoplasms. Therefore, it can be inferred that MMP enzymes are critical in the mechanism of invasion, angiogenesis, and metastasis of thyroid cancers.[36]

Control of proteolytic activity in ECM provides an opportunity to address common mechanisms of metastasis, angiogenesis, and tumor growth. It has been postulated that MMP inhibitors could be effective antitumor agents for the treatment of aggressive thyroid carcinomas.[37,38] Considerable evidence has accumulated regarding development of synthetic and specific MMP blockers as medications.[39] However, it seems that one of the most promising approaches to cancer would be targeting universal pathomechanisms involved in cancer growth and metastasis, such as encapsulating the tumor, strengthening of connective tissue, preventing angiogenesis, and blocking MMP activity. Rath and Pauling have suggested that lysine and lysine analogues are effective blockers of MMP-2 and MMP-9, and that they also strengthen the surrounding connective tissue to prevent matrix invasion and thus contribute to the encapsulation of the tumor.[17] Extracellular matrix integrity is dependant upon adequate collagen formation and stability, which is supported by lysine along with vitamin C and proline. Optimization of synthesis and structure of collagen fibrils depend on hydroxylation of proline and lysine residues in collagen fibrils. Ascorbic acid is essential for this hydroxylation and for regulating the collagen synthesis at a transcription level. Furthermore, lysine prevents cell migration by preventing collagen-digesting enzymes from binding to plasminogen-active sites, thereby blocking the activation of plasmin by plasminogen.[17] However, suboptimal levels of ascorbic acid and lysine are possible in various pathological stages and in deficient diets as these nutrients are not produced in the human body.

In this study, the dose dependant reduction of MMP-2, and also the significant reduction of Matrigel™ invasion by thyroid cancer cells SW 579, indicate that NM effectively blocks MMP-2 enzymes and supports collagen formation, thus preventing metastasis. This is especially significant in light of the fact that, at present, there is little or no cure available for thyroid cancer, and prolonging survival of the patient is the only goal of treatment combinations. While the death toll from thyroid cancer continues to mount every year, physicians are advised to combine two or more best-suited approaches for their patients.[16,40,41] Surgery is the most common choice of treatment for thyroid cancers; it does not, however, address metastasized cancers.[42] In addition to the inherent risks involved in any major operation, thyroid surgeries carry the additional risk of injury to the laryngeal nerve, leaving the person with permanent hoarseness of voice. Accidental removal of the parathyroid gland during surgery could lead to imbalances in calcium metabolism, with serious implications. In most cases, chemotherapy is used as a palliative measure. Although well-differentiated types, such as papillary and follicular, are the most common thyroid cancers, they have a high recurrence rate and are predisposing factors for anaplastic cancer. Even the most targeted radiotherapy indiscriminately attacks surrounding healthy cells as well as cancer, causing considerable cellular and ECM damage. In contrast to that, NM does not affect normal cells, even at high concentrations, indicating the safety of the combination. Cancer mortality results mainly from the tumor invading local tissues and metastasizing to vital organs, such as liver, lungs, and brain. As mentioned previously, degradation of ECM is hallmark of these events and is mediated by MMPs. Therefore, safe and effective inhibition of MMPs is the target of treatment.

CONCLUSIONS

While additional animal studies and clinical trials are required, the results of our study suggest that this formulation of Nutrient Mixture is a very good candidate for therapeutic evaluation in thyroid cancer as it inhibits MMP secretion and invasion of cancer cells, assuring safety for normal cells.

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